

INTRINSICALLY FLUORESCENT, SELF-MULTIMERIZING MHC  
FUSION PROTEINS AND COMPLEXES THEREOF

CROSS REFERENCE TO RELATED APPLICATION

5 This application claims the benefit of the  
filing date of U.S. provisional application serial no.  
60/261,448, filed January 12, 2001, the disclosure of  
which is incorporated herein by reference in its  
entirety.

FIELD OF THE INVENTION

10 The present invention relates to  
intrinsically fluorescent, self-multimerizing MHC  
fusion proteins and to complexes thereof, and to  
methods for using such complexes for the labeling,  
detection, enumeration, enrichment, isolation,  
15 depletion and activation of antigen specific T  
lymphocytes, particularly by flow cytometry.

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BACKGROUND OF THE INVENTION

Soluble tetramers of major histocompatibility complex (MHC) proteins, charged with specific peptide antigen and fluorescently labeled ("MHC tetramers"),  
5 have in the past five years proven extraordinarily useful in the detection, enumeration, characterization and purification of antigen-specific T lymphocytes. Applications of MHC class I tetramers have recently been reviewed in Doherty *et al.*, *Annu. Rev. Immunol.*  
10 18:561-92 (2000); Ogg *et al.*, *Immunol. Lett.* 66(1-3):77-80 (1999); Maini *et al.*, *Immunol. Today* 20(6):262-6 (1999); and Doherty, *Curr. Opin. Microbiol.* 1(4):419-22 (1998). Applications of MHC class II tetramers are discussed in Reichstetter *et al.*, *J.*  
15 *Immunol.* 165(12):6994-8 (2000); Kwok *et al.*, *J. Immunol.* 164(8):4244-9 (2000); Liu *et al.*, *Proc. Natl. Acad. Sci. USA* 97(26):14596-14601 (2000); Novak *et al.*, *J. Clin. Invest.* 104(12):R63-7 (1999); Crawford *et al.*, *Immunity* 8:675-682 (1998); and Kozono *et al.*, *Nature*  
20 369:151-154 (1994).

MHC tetramers bind to the T cell receptor ("TCR") of T lymphocytes in an antigen- and MHC-specific manner. See, e.g., Altman *et al.*, *Science* 274:94 (1996) and U.S. Patent No. 5,635,363.

25 Specificity of the staining reagent is conferred by both the MHC moiety and the peptide included within the tetrameric complex. Thus, tetramers comprising the extracellular domains of MHC class I  $\alpha$  chain molecules, complexed to  $\beta$ 2  
30 microglobulin and charged with antigenic peptides of about 9 amino acids, will bind to class I-restricted, typically CD8<sup>+</sup>, T cells specific for the charging

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peptide and MHC allele. Tetramers comprising four  
class II heterodimers - each heterodimer comprising the  
extracellular domains of MHC class II  $\alpha$  and class II  $\beta$   
chains - charged with antigenic peptides, will bind to  
5 class II-restricted, typically CD4<sup>+</sup>, T cells in an  
antigen-specific and MHC-restricted fashion.

Avidity sufficient to allow stable binding to  
the T lymphocyte results from the multimerization of  
the MHC/peptide complex. In order to maintain self-  
10 tolerance, the natural affinity of TCR for MHC/peptide  
is low, too low to permit use of a univalent  
MHC/peptide complex as an immunological staining  
reagent. Matsui et al., *Science* 254:1788-91 (1991);  
Matsui et al., *Proc. Natl. Acad. Sci. USA* 91:12862-6  
15 (1994). Multimerization increases the avidity of the  
MHC/peptide complex for TCR sufficiently to permit  
stable binding. Typically, multimerization is achieved  
by enzymatic biotinylation of a BirA substrate peptide  
engineered into the MHC chain; the biotinylated MHC  
20 chain thereafter binds avidin or streptavidin with a  
4:1 molar stoichiometry.

The MHC tetramer is rendered detectable by  
direct or indirect conjugation to a fluorophore.  
Typically, direct conjugation is accomplished by  
25 multimerizing the MHC/peptide molecules using a  
streptavidin or avidin molecule that has been prior-  
conjugated to a fluorophore. Such avidin and  
streptavidin proteins are commercially available from a  
variety of vendors (e.g., Becton Dickinson  
30 Immunocytometry Systems, San Jose, CA, USA; Biomed,  
Foster City, CA, USA; Ancell Corp., Bayport, MN USA;  
Southern Biotechnology Assocs., Inc., Birmingham, AL,  
USA). Indirect labeling can be performed using a

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fluorophore-conjugated antibody having specificity for the avidin/streptavidin moiety or for nonpolymorphic determinants of the multimerized MHC chain.

Although MHC tetramers have proven  
5 tremendously useful, certain aspects of their synthesis present problems.

Proper multimerization requires the controlled, stoichiometric, antecedent biotinylation of the MHC chain, and further requires the subsequent  
10 stoichiometric binding of the biotinylated chains to avidin or streptavidin. Inefficiency or imprecision at either or both of these steps affects yield and utility of the resulting tetrameric complex.

For example, inefficient biotinylation of the  
15 MHC chain - *i.e.*, failure to incorporate at least one biotin per MHC molecule - will lead to decreased yield of tetramers. Inefficient removal of unconjugated biotin from the reaction products, with carry-over of unconjugated biotin molecules into the multimerization  
20 reaction, can saturate avidin molecules and also decrease tetramer yield.

Biotinylation of MHC molecules at multiple sites can reduce the avidity of resulting tetrameric complexes. Early attempts at multimerization using  
25 chemical biotinylation failed, possibly because multiple and random chemical modification of MHC molecules led to randomized, and often inactive, orientation of the MHC/peptide moieties in the multimerized complex. Although enzymatic biotinylation  
30 has improved control of the biotinylation process, enzymatic modification is itself subject to well known vicissitudes, including dependence upon enzyme concentration, substrate concentration, substrate

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accessibility, enzyme activity, and the like; multiple enzymatic biotinylation can mimic the poor avidity observed with the early, chemically-biotinylated, complexes.

5           Biotinylation at multiple sites of the MHC chain, combined with obligate use of an extrinsic multimerizing moiety, such as avidin, also makes possible the spurious generation of higher order multimers during the multimerization reaction. If even  
10 a small number of MHC/peptide subunits contain two or more biotins, cross-linking of the avidin/streptavidin moiety can occur. Such higher order multimers can exhibit decreased avidity due to steric hindrance, and can conversely, in other circumstances, lead to higher  
15 order complexes having increased avidity. Variations in avidity, whether decreases or increases from the mean, can lead to variations in staining intensity that are unrelated to TCR density or antigen affinity. Higher order multimers can also produce a more intense  
20 fluorescent signal, further leading to confounding variations in staining intensity.

Conversely, carry-over of unconjugated biotin molecules into the multimerization reaction can lead to partial saturation of avidin/streptavidin moieties with  
25 unconjugated biotin, leading to formation of multimeric complexes having fewer than four MHC/peptide subunits. Such lower order complexes can demonstrate lower avidity for TCR, leading to variations in staining intensity that are unrelated to TCR density or  
30 antigen/MHC affinity.

The requirement for a separate fluorophore conjugation step also presents opportunities for yield

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loss and for unintended variation in either avidity for TCR or fluorescence intensity, or both.

In a related approach, MHC/Ig chimeras, rather than tetramers of MHC, are used to label antigen-specific T lymphocytes. Dal Porto *et al.*, *Proc. Natl. Acad. Sci. USA* 90:6671-6675 (1993); Greten *et al.*, *Proc. Natl. Acad. Sci. USA* 95:7568-7573 (1998); Hamad *et al.*, *J. Exp. Med.* 188:1633-1640 (1998); Schneck *et al.*, U.S. Pat. Nos. 6,015,884 and 6,140,113.

In class I chimeras, the extracellular domains of MHC class I  $\alpha$  chain are fused in-frame into the variable region of an IgG heavy chain. Under suitable oxidizing conditions, the heavy chain chimeras self-dimerize through disulfide bonds: dimerization confers avidity for TCR sufficient to permit use of the dimeric fusion protein as a T cell labeling reagent. As with MHC tetramers, the dimeric class I chimera molecule must be associated with  $\beta$ 2 microglobulin and charged with specific antigenic peptide to permit recognition of antigen-specific T cells; unlike MHC tetramers, the MHC/Ig chimeric molecules must additionally be associated prior to use with Ig light chains.

In class II MHC/Ig chimeras, the extracellular domains of MHC class II  $\alpha$  and  $\beta$  chains are separately fused to Ig heavy and light chain subunits, typically with the  $\beta$  subunit domains fused to IgG heavy chain and the class II  $\alpha$  domains fused to Ig light, typically  $\kappa$ , chain. As with MHC tetramers, the class II chimeric molecule must be charged with specific antigenic peptide to permit recognition of antigen-specific T cells; for stability, antigenic peptide can be fused directly to the N terminus of the

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class II  $\beta$  subunit, Kozono et al., *Nature* 369:151-154 (1994); Liu et al., *Proc. Natl. Acad. Sci. USA* 97:14596-14601 (2000).

Labeling of the dimeric chimeras is typically accomplished using a fluorophore-conjugated, anti-Ig, secondary antibody.

The self-dimerizing chimeras obviate the biotinylation step and use of an extrinsic multimerizing moiety required by MHC chimeras, with their attendant problems. The dimeric chimeras also more readily permit post-synthesis loading with antigenic peptide.

However, the dimeric chimeras are not without problems of their own.

First, the dimeric valency can cause lower avidity for TCR than would be obtained using tetramers, reducing or abrogating the ability to label low affinity TCRs.

Second, the additional requirement for Ig light chain association — whether native Ig light chains, as in the class I chimeras, or recombinant Ig light chain fusion proteins, as in the class II chimeras — obligates the coexpression of multiple recombinant expression constructs in a single host cell or, in the case of class I chimeras, use of a host cell line that constitutively expresses Ig light chain.

Third, although self-dimerization obviates the biotinylation step and reliance upon an extrinsic multimerizing moiety of MHC tetramers, potentially improving yield of multimers of known valency (complexity), the dimers can dissociate under reducing conditions.

Additionally, each of the strategies for rendering the dimeric chimera fluorescently detectable presents difficulties.

For example, use of secondary antibodies for  
5 visualization can make simultaneous detection of multiple antigens more difficult; this can be a significant problem when, as is often the case, the population of antigen-specific T cells is small, obligating use of multiple parameters in order  
10 accurately to detect and enumerate the cells. Direct chemical conjugation of fluorophore to the chimera, an alternative to use of secondary antibodies, can interfere with or even abrogate affinity interactions of the chimera with its cognate TCR. Direct chemical  
15 conjugation can also lead to chimeric molecules having varying molar ratios of label, making flow cytometric or other fluorescence-based analyses more problematic.

Thus, there is a need for reagents that can be used to label T lymphocytes based upon their antigen  
20 (and MHC) specificity that have sufficient avidity to permit stable binding to T lymphocytes via interactions with surface TCR, but that do not require biotinylation and extrinsic multimerization; there is a concurrent need for antigen-specific T cell labeling reagents that  
25 can be rendered fluorescent without requiring use of secondary antibodies or direct chemical conjugation to fluorophore.

The substrate-independent, intrinsically fluorescent green fluorescent protein from *Aequorea victoria* ("GFP") has been used in the flow cytometric  
30 ("FACS") analysis of cells that express GFP, or GFP fusions, inside the cell. Reviewed in Galbraith et al., "Flow cytometric analysis and FACS sorting of



cells based on GFP accumulation," *Methods Cell Biol.*  
58:315-41 (1999). GFP variants having characteristics  
improved for flow cytometry have been described, U.S.  
Pat. Nos. 6,090,919 and 5,804,387; Cormack *et al.*, *Gene*  
5 173:33-38 (1996). GFP and GFP variants have typically  
been used for internal labeling of cells, rather than  
for external labeling of cell surface structures.

A distant homologue of GFP, termed DsRed  
(also denominated drFP583), has recently been cloned  
10 from *Discosoma coral*, Matz *et al.*, *Nature Biotechnol.*  
17:969-973 (1999); vectors suitable for excision  
cloning, bacterial expression and mammalian expression  
of DsRed, alone or as a fusion protein, are now  
commercially available (Clontech Laboratories, Inc.,  
15 Palo Alto, CA, USA).

Native DsRed has an excitation (absorption)  
maximum of about 558 nm, and has an emission maximum of  
about 583 nm, a substantial red shift from the GFP  
emission maximum of about 509 nm. DsRed is thus  
20 readily excited by the standard 488 nm laser line  
routinely available in flow cytometers and has an  
emission spectrum readily distinguishable from  
autofluorescence background and from that of GFP,  
making DsRed a candidate for two-color flow cytometric  
25 applications in conjunction with GFP or FACS-optimized  
mutants of GFP.

Detailed studies of the structure and  
spectral properties of DsRed, Baird *et al.*, *Proc. Natl.*  
*Acad. Sci. USA* 97:11984-11989(2000); Gross *et al.*,  
30 *Proc. Natl. Acad. Sci. USA* 97:11990-11995 (2000);  
Heikal *et al.*, *Proc. Natl. Acad. Sci. USA* 97:11996-  
12001 (2000); Wall *et al.*, *Nature Structural Biol.*  
7:1133-1138 (2000), however, have identified two

characteristics that are said to militate against widespread use of DsRed in flow cytometric and other fluorescence analyses.

First, the red fluorescence of DsRed is slow  
5 to mature, requiring days to ripen fully from green to red, both *in vitro* and *in vivo*. A maturation time on the order of days will preclude attempts to use DsRed as a reporter of short-term gene expression or to track fusion proteins in organisms with short generation  
10 times or fast development. Baird et al., *Proc. Natl. Acad. Sci. USA* 97:11984-11989 (2000).

Second, DsRed appears to be an obligate, and self-multimerizing, tetramer. Baird et al., *Proc. Natl. Acad. Sci. USA* 97:11984-11989 (2000); Wall et  
15 al., *Nature Structural Biol.* 7:1133-1138 (2000); Gross et al., *Proc. Natl. Acad. Sci. USA* 97:11990-11995 (2000). Although oligomerization may be irrelevant to use of DsRed as a simple reporter of gene expression, it will present serious problems in most potential  
20 applications where DsRed would be fused to a host protein to report on the trafficking or interactions of the latter. Furthermore, many proteins in signal transduction are activated by oligomerization; fusion to DsRed could cause constitutive signaling. For host  
25 proteins that are already oligomeric, fusion to DsRed could either cause clashes of stoichiometry, steric conflicts of quaternary structures, or cross-linking into massive aggregates.

Given these "major drawbacks," "[m]any  
30 potential cell biological applications of DsRed will require suppression of the tetramerization and acceleration of the maturation." Baird et al., *Proc. Natl. Acad. Sci. USA* 97:11984-11989 (2000). "For most

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biotechnological applications, both the slow maturation and oligomerization of DsRed are undesirable properties that must be addressed through systematic mutagenesis."

Wall et al., *Nature Structural Biol.* 7:1133-1138

5 (2000). "The excellent brightness and stability of DsRed and many potential uses for a long-wavelength fluorescent protein provide ample justification for major efforts to remedy these remaining deficiencies."

Gross et al., *Proc. Natl. Acad. Sci. USA* 97:11990-11995

10 (2000).

#### SUMMARY OF THE INVENTION

It is an object of the present invention to provide reagents that can be used to label T lymphocytes based upon their antigen (and MHC)  
15 specificity, that have sufficient avidity for T lymphocytes to permit stable binding to surface TCR without requiring biotinylation and use of an extrinsic multimerizing moiety, and that can be rendered fluorescent without requiring use of secondary  
20 antibodies or direct chemical conjugation to fluorophore. It is a further object of the invention to provide methods for labeling, detecting, enumerating, enriching, isolating, depleting, and activating antigen-specific T lymphocytes using such  
25 reagents.

These, and other needs in the art, are satisfied by the present invention, which exploits a property — tetramerization — that is said to be disadvantageous in the newly discovered, intrinsically  
30 fluorescent, self-multimerizing fluorophore, DsRed, and which, by use of the resulting tetramer as an

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extracellular, rather than intracellular label, is unaffected by the other property said in the art to be disadvantageous in DsRed, the slow maturation of its protein fluorophore after translation.

5           Thus, in a first aspect, the invention provides a recombinant fusion protein, comprising: a GFP-like chromophore; at least one multimerization domain; and an MHC peptide-presenting moiety. Functionally, the fusion protein comprises means for  
10 fluorescing, the fluorescing means encoded entirely within the amino acid sequence of the protein; means for multimerizing; and means that are capable of contributing to presentation of a peptide antigen to an MHC-restricted T lymphocyte.

15           In a preferred embodiment, the fusion protein of the present invention comprises, from N-terminus to C-terminus, contiguous  $\alpha 1$ ,  $\alpha 2$  and  $\alpha 3$  domains drawn from a chosen MHC class I  $\alpha$  subunit, followed in frame by substantially all of DsRed, which provides both the  
20 GFP-like fluorophore and two distinct multimerization domains. This preferred fusion protein spontaneously tetramerizes in solution.

          When properly multimerized and charged with peptide, the fusion proteins of the present invention  
25 can be used, without further direct or indirect conjugation to an exogenous fluorophore, fluorescently to label T lymphocytes based upon their antigen (and MHC) specificity; the reagents of the present invention can thus be used in all methods for which MHC tetramers  
30 and MHC/Ig chimeric fusion proteins are presently used.

          The fusion proteins of the present invention are typically produced by recombinant expression in host cells. Thus, in further aspects, the present

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invention includes nucleic acids that encode the above-described fusion proteins, and their complements, vectors comprising the nucleic acids, vectors for expressing the fusion proteins in host cells, host  
5 cells containing episomal or integrated vectors capable of expressing the fusion proteins of the present invention, and host cells containing any one of the nucleic acids, recombinant vector, expression vector, and fusion proteins of the present invention in one or  
10 more internal cellular compartments.

In other aspects, the present invention provides multimeric complexes that include, as plural subunits, the fusion proteins of the present invention. When properly complexed with appropriate soluble  
15 protein partners (e.g.,  $\beta 2$  microglobulin for fusion proteins having an class I MHC peptide-presenting moiety) and charged with peptide, these intrinsically fluorescent multimeric complexes can be used to label T lymphocytes based upon their antigen (and MHC)  
20 specificity.

In the simplest such aspect, the invention provides an intrinsically fluorescent, multimeric protein complex, comprising: a plurality of subunits, the subunits having the quaternary formula in the  
25 complex of  $(F)_n$ , wherein F is a fusion protein according to the present invention and n is an integer greater than 1. Where the multimerization domain of the fusion protein is provided by DsRed or related fusion protein, the complex typically assembles with tetrameric valency  
30 (i.e.,  $n = 4$ ).

In a related aspect, the invention further provides an intrinsically fluorescent, multimeric protein complex, comprising: a plurality of subunits,

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the subunits having the quaternary formula in the complex of  $(F_1S_1)_n$ , wherein F is a recombinant fusion protein according to the present invention, S is a soluble protein, and n is an integer greater than 1.

5               S is selected from the group consisting of  $\beta 2$  microglobulin, class II  $\beta$  MHC peptide-presenting soluble derivatives, and class II  $\alpha$  MHC peptide-presenting soluble derivatives. S is  $\beta 2$  microglobulin when F includes a class I  $\alpha$  MHC peptide-presenting  
10 moiety, S is a class II  $\beta$  MHC peptide-presenting soluble derivative when F includes a class II  $\alpha$  MHC peptide-presenting moiety, and S is a class II  $\alpha$  MHC peptide presenting soluble derivative when F includes a class II  $\beta$  MHC peptide-presenting moiety.

15               The multimeric complex, properly assembled with soluble protein (i.e., with  $(F_1S_1)_n$  quaternary stoichiometry), can thereafter be charged with specific peptide, providing an intrinsically fluorescent reagent that is useful for detectable labeling of T lymphocytes  
20 based upon their antigen specificity.

              Thus, in another aspect, the invention provides an intrinsically fluorescent, multimeric protein complex for labeling T lymphocytes based upon the specificity of their antigen receptor. The complex  
25 of this aspect of the invention comprises a plurality of subunits, the subunits having the quaternary formula in the complex of  $(F_1S_1P_1)_n$ , where F, as above, is a recombinant fusion protein of the present invention, S is a soluble protein as above-described, P is a peptide  
30 antigen, and n is an integer greater than 1.

              The peptide antigen will be bound, as in MHC tetramers and MHC/Ig chimeras, by the MHC peptide-presenting domains of the multimeric complex of the

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present invention: in a class I MHC intrinsically  
fluorescent multimer, by the  $\alpha 1$  and  $\alpha 2$  domains of the  
class I  $\alpha$  MHC peptide presenting moiety of the fusion  
protein subunit; in a class II multimer, by the  $\alpha 1$  and  
5  $\beta 1$  domains of the MHC peptide-presenting moiety and  
soluble derivatives of the complex.

Functionally, the intrinsically fluorescent,  
multimeric protein complex for labeling T lymphocytes  
according to the specificity of their antigen  
10 receptors, comprises: means for fluorescing, the means  
being encoded entirely within an amino acid sequence of  
at least one subunit of the multimeric complex; and  
means for binding to a T lymphocyte according to the  
specificity of its antigen receptor.

15 Once charged with a given peptide antigen,  
the intrinsically fluorescent complexes of the present  
invention can bind to T lymphocytes having antigen  
receptors that are specific for the given peptide and  
MHC peptide-presenting domains of the multimeric  
20 complex. Because the peptide antigens and MHC peptide-  
presenting domains of the complexes of the present  
invention can both be varied, the intrinsically  
fluorescent multimeric complexes of the present  
invention can be used, in general, fluorescently to  
25 label a near infinite variety of T lymphocytes based  
upon the antigen (and MHC) specificity of their antigen  
receptors.

It is, therefore, another aspect of the  
present invention to provide methods for using the  
30 multimeric complexes of the present invention  
detectably to label (stain) T lymphocytes based upon  
the specificity of their antigen receptors.

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In a first embodiment, the method comprises contacting a T lymphocyte to be labeled with an intrinsically fluorescent multimeric complex of the present invention, the complex having peptide antigen  
5 and MHC peptide-presenting domains for which the antigen receptor of the T lymphocyte is specific, for a time and under conditions sufficient to permit detectable binding of the complex to the T lymphocyte.

Typically, the T lymphocytes to be labeled  
10 are present within a heterogeneous sample of cells, and the goal of labeling is to detect, and often to enumerate, the antigen specific T lymphocytes within this population.

Thus, in another aspect, the present  
15 invention provides methods for detecting, in a sample of cells, T lymphocytes that are specific for a chosen antigen. The method comprises contacting the sample with an intrinsically fluorescent multimeric complex according to the present invention, wherein the peptide  
20 antigen of the complex is the chosen antigen and the MHC presenting domains of the complex are those for which the T lymphocytes desired to be detected will be restricted, for a time and under conditions sufficient to permit detectable binding of the complex to T  
25 lymphocytes in the sample that are specific for the chosen antigen and MHC; and then detecting specific T lymphocytes in the sample by the fluorescence of the complex bound thereto. In a related aspect, the method further comprises enumerating the antigen-specific T  
30 lymphocytes so detected.

Depending upon the instrument used, detection of antigen-specific T lymphocytes can be coupled directly or indirectly to their sorting, thus



providing, in other aspects of the invention, methods for enriching a sample in, and for depleting a sample of, T lymphocytes that are specific for a chosen antigen.

5                   In general, the methods of this aspect of the invention comprise contacting the sample with an intrinsically fluorescent multimeric complex of the present invention, wherein the peptide antigen of the complex is the chosen antigen and the MHC presenting  
10 domains of the complex are those for which the T lymphocytes desired to be enriched or depleted will be restricted, for a time and under conditions sufficient to permit detectable binding of the complex to T lymphocytes in the sample that are specific for the  
15 chosen antigen and MHC. After binding, labeled T lymphocytes are enriched or depleted based upon the fluorescence of the complex bound thereto.

Such methods are conveniently performed using a fluorescence activated cell sorter: sorting based at  
20 least in part upon fluorescence from the multimeric complex of the present invention directly depletes the sample from which the cells are removed and enriches the aliquot into which the cells are placed.

The multimeric complex compositions of the present invention can usefully be provided in the form  
25 of kits that facilitate the practice of the methods of the present invention. Thus, in another aspect, the present invention provides kits comprising the intrinsically fluorescent multimeric complexes of the  
30 present invention.

In one series of embodiments, the kits of the present invention include, as separate compositions, an intrinsically fluorescent  $(F_1S_1)_n$  multimer of the

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present invention, and an antigenic peptide; as noted above, the choice of peptide will depend upon the specificity desired for the labeling reagent. In another series of embodiments, the multimer is prior-  
5 charged with peptide. Optionally, but advantageously, included in either of these series of kits as separate compositions can be any of fluorophore-conjugated antibodies to pan-T or T cell subsetting antigens, fluorophore-conjugated antibodies specific for T cell  
10 activation antigens, and red cell lysing reagents.

#### BRIEF DESCRIPTION OF THE DRAWINGS

The above and other objects and advantages of the present invention will be apparent upon consideration of the following detailed description  
15 taken in conjunction with the accompanying drawings, in which like characters refer to like parts throughout, and in which:

FIG. 1 schematizes construction of an H2Ld MHC class I-DsRed recombinant fusion construct  
20 according to the present invention;

FIG. 2 schematizes a dual promoter expression vector containing the H2Ld-DsRed recombinant fusion construct according to the present invention;

FIG. 3 is a photograph of an agarose gel  
25 showing the expected 1.5kD H2Ld-DsRed recombinant fusion construct insert upon release from expression vector plasmids digested with NdeI and NotI;

FIG. 4 is an automatic DNA sequencer trace further confirming the correct DNA sequence at the junction between the expression vector backbone and the H2Ld insert;

5           FIG. 5 is a photograph of a stained SDS-PAGE gel showing induction by IPTG of H2Ld-DsRed recombinant fusion protein in transformed *E. coli*;

FIG. 6 is a photograph of a stained SDS-PAGE gel showing induction by IPTG of H2Ld-DsRed recombinant fusion protein in different transformed *E. coli* strains;

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FIG. 7 is a photograph of a stained SDS-PAGE gel showing expression of H2Ld-DsRed recombinant fusion protein in insect cells infected with H2Ld-DsRed recombinant baculovirus;

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FIG. 8 is a photograph by conventional microscopy of non-infected Sf9 cells grown three days in culture;

FIG. 9 is a photograph by fluorescent microscopy using a rhodamine filter of non-infected Sf9 cells grown three days in culture;

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FIG. 10 is a photograph by conventional microscopy of Sf9 cells infected with the H2Ld-DsRed recombinant baculovirus grown three days in culture post-infection;

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FIG. 11 is a photograph by fluorescent microscopy using a rhodamine filter of Sf9 cells infected with the H2Ld-DsRed recombinant baculovirus grown three days in culture post-infection, showing red fluorescence;

FIG. 12 is a photograph by conventional microscopy of Tni cells infected with the H2Ld-DsRed recombinant baculovirus grown three days in culture post-infection; and

FIG. 13 is a photograph by fluorescent microscopy using a rhodamine filter of Tni cells infected with the H2Ld-DsRed recombinant baculovirus grown three days in culture post-infection, showing red fluorescence.

## DETAILED DESCRIPTION OF THE INVENTION

### Definitions

As used herein, the terms set forth with particularity below have the following definitions. If not otherwise defined in this section, all terms used herein have the meaning commonly understood by a person skilled in the arts to which this invention belongs.

**"GFP-like chromophore"** means an intrinsically fluorescent protein moiety comprising an 11-stranded  $\beta$ -barrel ( $\beta$ -can) with a central  $\alpha$ -helix, the central  $\alpha$ -helix having a conjugated  $\pi$ -resonance system that includes two aromatic ring systems and the bridge between them. By "intrinsically fluorescent" is meant that the GFP-like chromophore is entirely encoded by

its amino acid sequence and can fluoresce without requirement for cofactor or substrate.

"MHC peptide-presenting moiety", "MHC peptide-presenting soluble derivative", and "MHC peptide-presenting domains" all refer to portions of a major histocompatibility complex protein chain that lack a transmembrane domain (and are thus "soluble") and that can participate in presentation of peptide antigen to a T cell receptor. The term "MHC peptide-presenting moiety" is used when the soluble MHC portion is included within a fusion protein; the variant "MHC peptide-presenting soluble derivative" is used when the protein is expressed unfused; the phrase "MHC peptide-presenting domains" is used generically to both contexts.

Class I  $\alpha$  MHC peptide-presenting domains include substantially all of the  $\alpha 1$ ,  $\alpha 2$  and  $\alpha 3$  domains of an MHC class I molecule; by "substantially all" is meant that deletions therefrom and substitutions thereof are permissible only when they do not interfere with ability of the  $\alpha 3$  domain to form an intradomain disulfide bond or the ability of  $\alpha 1$  and  $\alpha 2$  domains to form alpha helices properly positioned to bind peptide.

Class II  $\alpha$  MHC peptide-presenting domains include substantially all of the  $\alpha 1$  and  $\alpha 2$  domains of an MHC class II  $\alpha$  chain molecule; by "substantially all" is meant that deletions therefrom or substitutions thereof are permissible only when they do not interfere with the ability of  $\alpha 2$  domain to form intrachain disulfide bonds or with the ability of the  $\alpha 1$  domain to form a peptide binding  $\alpha$  helix.

Class II  $\beta$  MHC peptide-presenting domains include substantially all of the  $\beta 1$  and  $\beta 2$  domains of

an MHC class II  $\beta$  chain molecule. By "substantially all" is meant that deletions therefrom or substitutions thereof are permissible only when they do not interfere with the ability of the  $\beta 2$  domain to form intrachain  
5 disulfide bonds or of the  $\beta 1$  domain to form peptide binding  $\alpha$  helix.

### Fusion proteins

In a first aspect, the present invention provides a recombinant fusion protein that, when  
10 properly multimerized and charged with peptide, can be used, without further direct or indirect conjugation to an exogenous fluorophore, fluorescently to label T lymphocytes based upon their antigen (and MHC) specificity; the reagents of the present invention can  
15 thus be used in all methods for which MHC tetramers and MHC/Ig chimeric fusion proteins are presently used, including peptide antigen-specific activation of T-cells.

The fusion protein of the present invention  
20 comprises a GFP-like chromophore, at least one multimerization domain, and an MHC peptide-presenting moiety.

A GFP-like chromophore is an intrinsically fluorescent protein moiety. By "intrinsically  
25 fluorescent" is meant that the GFP-like chromophore is entirely encoded by its amino acid sequence and can fluoresce without requirement for cofactor or substrate.

Structurally, the GFP-like chromophore  
30 comprises an 11-stranded  $\beta$ -barrel ( $\beta$ -can) with a central  $\alpha$ -helix, the central  $\alpha$ -helix having a

conjugated  $\pi$ -resonance system that includes two aromatic ring systems and the bridge between them. The  $\pi$ -resonance system is created by autocatalytic cyclization among amino acids; cyclization proceeds  
5 through an imidazolinone intermediate, with subsequent dehydrogenation by molecular oxygen at the C $\alpha$ -C $\beta$  bond of a participating tyrosine.

The GFP-like chromophore can be selected from GFP-like chromophores found in naturally occurring  
10 proteins, such as *A. victoria* GFP (GenBank accession number AAA27721), *Renilla reniformis* GFP, FP583 (GenBank accession no. AF168419) (DsRed), FP593 (AF272711), FP483 (AF168420), FP484 (AF168424), FP595 (AF246709), FP486 (AF168421), FP538 (AF168423), and  
15 FP506 (AF168422), and need include only so much of the native protein as is needed to retain the chromophore's intrinsic fluorescence. Methods for determining the minimal domain required for fluorescence are known in the art. Li et al., "Deletions of the *Aequorea victoria*  
20 Green Fluorescent Protein Define the Minimal Domain Required for Fluorescence," *J. Biol. Chem.* 272:28545-28549 (1997).

Alternatively, the GFP-like chromophore can be selected from GFP-like chromophores modified from  
25 those found in nature. Typically, such modifications are made to improve recombinant production in heterologous expression systems (with or without change in protein sequence), to alter the excitation and/or emission spectra of the native protein, to facilitate  
30 purification, to facilitate or as a consequence of cloning, or are a fortuitous consequence of research investigation.

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The methods for engineering such modified GFP-like chromophores and testing them for fluorescence activity, both alone and as part of protein fusions, are well-known in the art. Early results of these efforts are reviewed in Heim *et al.*, *Curr. Biol.* 6:178-182 (1996), incorporated herein by reference in its entirety; a more recent review, with tabulation of useful mutations, is found in Palm *et al.*, "Spectral Variants of Green Fluorescent Protein," in Green  
10 Fluorescent Proteins, Conn (ed.), *Methods Enzymol.* vol. 302, pp. 378 - 394 (1999), incorporated herein by reference in its entirety. A variety of such modified chromophores are now commercially available and can readily be used in the fusion proteins of the present  
15 invention.

For example, EGFP ("enhanced GFP"), Cormack *et al.*, *Gene* 173:33-38 (1996); U.S. Pat. Nos. 6,090,919 and 5,804,387, is a red-shifted, human codon-optimized variant of GFP that has been engineered for brighter  
20 fluorescence, higher expression in mammalian cells, and for an excitation spectrum optimized for use in flow cytometers. EGFP can usefully contribute a GFP-like chromophore to the fusion proteins of the present invention. A variety of EGFP vectors, both plasmid and  
25 viral, are available commercially (Clontech Labs, Palo Alto, CA, USA), including vectors for bacterial expression, vectors for N-terminal protein fusion expression, vectors for expression of C-terminal protein fusions, and for bicistronic expression.

30 Toward the other end of the emission spectrum, EBFP ("enhanced blue fluorescent protein") and BFP2 contain four amino acid substitutions that shift the emission from green to blue, enhance the



brightness of fluorescence and improve solubility of  
the protein, Heim et al., *Curr. Biol.* 6:178-182 (1996);  
Cormack et al., *Gene* 173:33-38 (1996). EBFP is  
optimized for expression in mammalian cells whereas  
5 BFP2, which retains the original jellyfish codons, can  
be expressed in bacteria; as is further discussed  
below, the host cell of production does not affect the  
utility of the resulting fusion protein. The GFP-like  
chromophores from EBFP and BFP2 can usefully be  
10 included in the fusion proteins of the present  
invention, and vectors containing these blue-shifted  
variants are available from Clontech Labs (Palo Alto,  
CA, USA).

Analogously, EYFP ("enhanced yellow  
15 fluorescent protein"), also available from Clontech  
Labs, contains four amino acid substitutions, different  
from EBFP, Ormö et al., *Science* 273:1392-1395 (1996),  
that shift the emission from green to yellowish-green.  
Citrine, an improved yellow fluorescent protein mutant,  
20 is described in Heikal et al., *Proc. Natl. Acad. Sci.*  
*USA* 97:11996-12001 (2000). ECFP ("enhanced cyan  
fluorescent protein") (Clontech Labs, Palo Alto, CA,  
USA) contains six amino acid substitutions, one of  
which shifts the emission spectrum from green to cyan.  
25 Heim et al., *Curr. Biol.* 6:178-182 (1996); Miyawaki et  
al., *Nature* 388:882-887 (1997). The GFP-like  
chromophore of each of these GFP variants can usefully  
be included in the fusion proteins of the present  
invention.

30 The GFP-like chromophore can also be drawn  
from other modified GFPs, including those described in  
U.S. Pat. Nos. 6,124,128; 6,096,865; 6,090,919;  
6,066,476; 6,054,321; 6,027,881; 5,968,750; 5,874,304;

5,804,387; 5,777,079; 5,741,668; and 5,625,048, the disclosures of which are incorporated herein by reference in their entireties.

The fusion proteins of the present invention further include at least one multimerization domain. If plural multimerization domains are present, the domains need not be identical to one another. The multimerization domains can function either to effect homomultimerization or heteromultimerization, and can contribute to noncovalent or covalent association of the individual subunits.

For example, the multimerization domain can usefully be drawn from existing, self-dimerizing, MHC/Ig chimeras, which are further described in Dal Porto et al., *Proc. Natl. Acad. Sci. USA* 90:6671-6675 (1993); Greten et al., *Proc. Natl. Acad. Sci. USA* 95:7568-7573 (1998); Hamad et al., *J. Exp. Med.* 188:1633-1640 (1998); Schneck et al., U.S. Pat. Nos. 6,015,884 and 6,140,113, the disclosures of which are incorporated herein by reference in their entireties. The multimerizing moiety of these chimeras includes an IgG Fc region, which is capable of disulfide-linked homodimerization.

Other protein multimerization domains are well known, and include, for example, leucine zipper domains.

The multimerization domain(s) can also usefully be provided by the same protein that provides the GFP-like chromophore.

30 DsRed exists as an obligate tetramer, even in dilute solution, and has not been observed to monomerize without protein denaturation, Gross et al., *Proc. Natl. Acad. Sci. USA* 97:11990-11995 (2000); Wall

et al., *Nature Structural Biol.* 7:1133-1138 (2000), a property that has been viewed in the art as disadvantageous and warranting interventional ablation. In the present invention, this disadvantageous property  
5 proves surprisingly useful, allowing DsRed to provide both the GFP-like chromophore and the multimerization domains to the fusion protein of the present invention; with such multimerization domains, the fusion proteins of the present invention readily assemble into soluble  
10 MHC/peptide complexes with the tetrameric valency, and thus avidity characteristics, of known MHC tetramers.

DsRed contains two dissimilar multimerization domains, both of which appear necessary to its tetramerization. The smaller domain shows  
15 characteristics typical of many high-affinity protein-protein interaction surfaces, and is perhaps specifically tuned for homooligomeric interaction; the larger interface is dramatically different in chemical character, and has characteristics observed at  
20 oligomerization interfaces that seem to require broader tuning of substrate specificity.

The two multimerization domains of DsRed can advantageously be used in the fusion proteins of the present invention in association with the native DsRed  
25 GFP-like chromophore, providing the excitation (absorption) maximum of about 558 nm, emission maximum of about 583 nm, strong resistance to photobleaching and strong pH independence of the native DsRed protein.

Alternatively, the multimerization domains of  
30 DsRed can be used with GFP-like chromophores modified from the native DsRed chromophore. For example, the green-only DsRed mutant K83R (lysine to arginine at residue 83, numbering as in Baird et al., *Proc. Natl.*

Acad. Sci. USA 97:11984-11989 (2000)) fails to mature  
to red fluorescence, yet tetramerizes, and thus will  
prove useful in the fusion proteins of the present  
invention, providing tetramers with emission spectrum  
5 readily distinguishable from those of tetramers  
composed of fusions using the native DsRed fluorophore.  
Conversely, DsRed mutant K83M has a 602 nm emission  
maximum, with relatively little residual green  
fluorescence, Baird et al., *Proc. Natl. Acad. Sci. USA*  
10 97:11984-11989 (2000)), and thus will provide further  
spectral separation from green- and yellow-emitting  
fluorophores. Other DsRed chromophore mutants can  
readily be created, expressed, and screened for  
spectral and oligomerization characteristics,  
15 essentially as described in Baird et al., *Proc. Natl.*  
*Acad. Sci. USA* 97:11984-11989 (2000).

Additionally, although DsRed is only 22%  
identical in sequence to GFP, the overall structure of  
the chromophore is strikingly conserved as between  
20 these distant homologues. Thus, the chromophore from  
*A. victoria* GFP and its many known variants, including  
EGFP, EYFP, EBFP, and Citrine, can readily be  
substituted for the DsRed chromophore, altering the  
spectral properties of the protein while retaining the  
25 DsRed multimerization domains. This can advantageously  
be done by engineering into existing DsRed vectors  
restriction sites that flank the DsRed chromophore and  
that are suitable for excision and in-frame replacement  
of the DsRed GFP-like chromophore by cassettes  
30 containing the sequence coding for other GFP-like  
chromophores.

Such DsRed/GFP chimeras are thus suitable for  
and can advantageously be used for inclusion in the

fusion proteins of the present invention. As further discussed below, fusion proteins of the present invention that have the DsRed multimerizing moieties and differing GFP-like chromophores can be assembled  
5 into heteromeric tetramers in which the respective chromophores participate as donor and acceptor in fluorescence resonance energy transfer ("FRET") reactions.

Multimerization domains can also be provided  
10 by fluorescent protein homologues of DsRed or A. victoria GFP.

For example, *Renilla reniformis* GFP is believed to be an obligate dimer. Ward, in Green Fluorescent Protein: Properties, Applications, and  
15 Protocols, eds. Chalfie & Kain (Wiley, New York) (1998). The *Renilla* GFP multimerization domain can usefully be used in the fusion proteins of the present invention to permit self-assembly of complexes with the dimeric valency, and thus avidity characteristics, of existing  
20 MHC/Ig chimeras.

Further, Wall et al., *Nature Structural Biol.* 7:1133-1138 (2000), report that oligomerization appears to be a general property of the DsRed-like fluorescent proteins, such as FP593, FP483, FP484, FP595, FP486,  
25 FP538 and FP506. Thus, each of these fluorescent proteins can usefully contribute one or more multimerization domains to the fusion protein of the present invention. As with use of the DsRed multimerization domains, the fusion protein of the  
30 present invention can include the GFP-like chromophore of the fluorescent protein contributing the multimerization domain(s), a mutational variant thereof, or, alternatively, can include the GFP-like

chromophore of another, homologous, fluorescent protein or a variant thereof.

The fusion proteins of the present invention further include an MHC peptide-presenting moiety.

5           The MHC peptide-presenting moiety is a soluble portion of a major histocompatibility complex protein chain that is capable of participating in, but may not itself be sufficient for, antigen presentation to a T cell receptor.

10           The MHC peptide-presenting moiety can be derived from MHC class I, MHC class II, or nonclassical MHC molecules.

          If derived from class I, the MHC peptide-presenting moiety will be derived from the MHC class I  
15    $\alpha$  chain and will include the  $\alpha 1$ ,  $\alpha 2$  and  $\alpha 3$  domains; permissible deletions therefrom or substitutions thereof cannot interfere with ability of the  $\alpha 3$  domain to form an intradomain disulfide bond or the ability of  $\alpha 1$  and  $\alpha 2$  domains to form alpha helices properly  
20   positioned to bind peptide.

          If derived from class II, the MHC peptide-presenting moiety of the fusion protein of the present invention will be derived from either the MHC class II  $\alpha$  or  $\beta$  chain; multimeric complexes of the present  
25   invention, useful for detecting class II restricted antigen-specific T cell labeling, will, as further discussed below, include both fusion proteins having a class II  $\alpha$ - and a class II  $\beta$ -derived MHC peptide-presenting moiety.

30           If derived from class II  $\alpha$  chain, the MHC peptide presenting moiety of the fusion protein of the present invention will include  $\alpha 1$  and  $\alpha 2$  domains; permissible deletions therefrom or substitutions

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thereof must not interfere with ability of  $\alpha 2$  domain to form intrachain disulfide bonds or with the ability of the  $\alpha 1$  domain to form a peptide binding  $\alpha$  helix. If derived from class II  $\beta$  chain, the MHC peptide  
5 presenting moiety of the fusion protein of the present invention will include  $\beta 1$  and  $\beta 2$  domains; permissible deletions therefrom or substitutions thereof must not interfere with ability of the  $\beta 2$  domain to form intrachain disulfide bonds or of the  $\beta 1$  domain to form  
10 peptide binding  $\alpha$  helix.

If derived from a nonclassical MHC molecule, the domains to be included will depend upon whether the molecule bears greater sequence homology to classical class I or to class II MHC molecules.

15 The MHC peptide-presenting moiety will lack the transmembrane region of its respective parent MHC molecule; typically, the MHC peptide-presenting moiety will also lack the cytoplasmic region of the respective MHC molecule.

20 The MHC peptide presenting moiety of the fusion protein of the present invention can be derived from the MHC molecules of any mammalian or avian species. For use in multimeric complexes for labeling of antigen-specific T cells, the MHC peptide presenting  
25 moiety will advantageously be drawn from the MHC molecules of the species whose T cells are to be detected.

Thus, the MHC peptide presenting moiety of the fusion protein of the present invention can  
30 advantageously be derived from human and other primate MHC molecules, from rodent, particularly mouse, rat, hamster, and guinea pig MHC, and from rabbit, bovine, canine, feline, and equine MHC. The MHC peptide

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presenting moiety of the fusion protein of the present invention can thus usefully be drawn from the human class II subunits HLA-DP $\alpha$ , HLA-DP $\beta$ , HLA-DQ $\alpha$ , HLA-DQ $\beta$ , HLA-DR $\alpha$  and HLA-DR $\beta$ , the human class I subunits HLA-A, HLA-B, HLA-C, murine class II subunits I-A $\alpha$ , I-A $\beta$ , I-E $\alpha$ , I-E $\beta$ , and murine class I subunits H-2K, H-2D, and H-2L.

The MHC peptide presenting moiety of the fusion protein of the present invention can be substantially the same as, and can be identical to, the MHC-derived portions of MHC tetramers, further described in Doherty et al., *Annu. Rev. Immunol.* 18:561-92 (2000); Ogg et al., *Immunol. Lett.* 66(1-3):77-80 (1999); Maini et al., *Immunol. Today* 20(6):262-6 (1999); Doherty, *Curr. Opin. Microbiol.* 1(4):419-22 (1998); Reichstetter et al., *J. Immunol.* 165(12):6994-8 (2000); Kwok et al., *J. Immunol.* 164(8):4244-9 (2000); Liu et al., *Proc. Natl. Acad. Sci. USA* 97(26):14596-14601 (2000); Novak et al., *J. Clin. Invest.* 104(12):R63-7 (1999); Crawford et al., *Immunity* 8:675-682 (1998); Kozono et al., *Nature* 369:151-154 (1994); Altman et al., *Science* 274:94 (1996); and Altman et al., U.S. Patent No. 5,635,363.

The MHC peptide presenting moiety of the fusion protein of the present invention can be substantially the same as, and can be identical to, the MHC-derived portions of MHC/Ig chimeras, further described in Dal Porto et al., *Proc. Natl. Acad. Sci. USA* 90:6671-6675 (1993); Greten et al., *Proc. Natl. Acad. Sci. USA* 95:7568-7573 (1998); Hamad et al., *J. Exp. Med.* 188:1633-1640 (1998); Schneck et al., U.S. Pat. Nos. 6,015,884 and 6,140,113, the disclosures of



all of which are incorporated herein by reference in their entireties.

Other MHC molecules that can contribute MHC peptide-presenting moieties can be found in Sequences of Proteins of Immunological Interest, Kabat *et al.*, (eds.), 5th ed., U.S. Dept. of Health and Human Services, Public Health Service, National Institutes of Health (1991), the disclosure of which is incorporated herein by reference in its entirety, and The Kabat Database of Sequences of Proteins of Immunological Interest, [http:// immuno.bme.nwu.edu/](http://immuno.bme.nwu.edu/), further described in Johnson *et al.*, *Nucl. Acids Res.* 29: 205-206 (2001).

Typically, but not invariably, the MHC peptide presenting moiety will be located N-terminal to both the GFP-like chromophore and the multimerization domains of the fusion protein of the present invention. The relative position of the chromophore and multimerization domains, in turn, will depend in large part upon their source.

For example, where the multimerization domain (and perhaps also the MHC peptide presenting moiety) is drawn from an existing MHC/Ig chimera, the GFP-like chromophore is advantageously fused to the C terminus of the existing MHC/Ig chimera. As another example, where the GFP-like chromophore and multimerization domains are drawn from a single escent protein, such as DsRed, the relative positions will be determined by their relative positions in the native protein. Relative positioning of the multimerization domain(s) and GFP-like chromophore may also depend upon cloning considerations, the solutions to which are well within the knowledge of the skilled molecular biologist.

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In a preferred embodiment, the fusion protein of the present invention comprises, from N-terminus to C-terminus, contiguous  $\alpha 1$ ,  $\alpha 2$  and  $\alpha 3$  domains drawn from a chosen MHC class I  $\alpha$  subunit, followed in frame by  
5 substantially all of DsRed, which provides both the GFP-like chromophore and two distinct multimerization domains. The fusion protein spontaneously tetramerizes in solution. The resulting tetramers can be used, after complexing with  $\beta 2$ -microglobulin and charging  
10 with peptide, and without further labeling, in all uses for which classic class I MHC tetramers and class I MHC/Ig chimeras have proven useful.

The fusion protein of the present invention can also usefully include, N-terminal to the MHC-  
15 presenting moiety, the antigenic peptide itself; this is particularly useful in fusions that include an MHC class II  $\beta$  subunit peptide presenting moiety. Kozono *et al.*, *Nature* 369:151-154 (1994); Liu *et al.*, *Proc. Natl. Acad. Sci. USA* 97:14596-14601 (2000),  
20 incorporated herein by reference in their entireties.

The fusion protein can also advantageously include, at either N-terminus or C-terminus, a sequence useful for purification. Where present directly adjacent to the MHC peptide-presenting moiety, this  
25 fused tag is preferentially selectively removable.

For example, the fusion protein can include a polyhistidine tag, permitting purification by immobilized metal affinity chromatography, for example using NiNTA resin (Qiagen Inc., Valencia, CA, USA) or  
30 TALON™ resin (cobalt immobilized affinity chromatography medium, Clontech Labs, Palo Alto, CA, USA). As another example, the fusion protein can include a chitin-binding tag and self-excising intein,

permitting chitin-based purification with self-removal  
of the fused tag (IMPACT™ system, New England Biolabs,  
Inc., Beverly, MA, USA). Alternatively, the fusion  
protein can include a calmodulin-binding peptide tag,  
5 permitting purification by calmodulin affinity resin  
(Stratagene, La Jolla, CA, USA), or a specifically  
excisable fragment of the biotin carboxylase carrier  
protein, permitting purification of *in vivo*  
biotinylated protein using an avidin resin and  
10 subsequent tag removal (Promega, Madison, WI, USA).

In alternative embodiments, the fusion  
protein can also advantageously include a protein  
spacer fused in frame between the MHC-presenting moiety  
and any of the GFP-like chromophore and multimerization  
15 domain or domains; the spacer provides a flexible link  
between the MHC-presenting moiety and other components  
of the fusion protein. The linker sequence may appear  
once, or as multiple tandem repeats of the same  
sequence. Suitable amino acid sequences that will  
20 provide a flexible link between the MHC-presenting  
moiety and DsRed, and techniques for inserting such a  
spacer into the fusion construct, are within the  
knowledge of the skilled artisan.

#### Nucleic acids, vectors, and host cells

25 The fusion proteins of the present invention  
are typically produced by recombinant expression in  
host cells. Thus, in further aspects, the present  
invention includes nucleic acids that encode the above-  
described fusion proteins, vectors comprising the  
30 nucleic acids, vectors for expressing the fusion  
proteins in host cells, host cells containing episomal

or integrated vectors capable of expressing the fusion proteins of the present invention, and host cells containing the fusion proteins of the present invention in one or more internal cellular compartments.

5           The nucleic acids of the present invention comprise sequences that encode the fusion protein or that are complementary in sequence to those that encode the fusion protein. The nucleic acids (or respective complements thereof) can encode the fusion protein as a  
10 single open reading frame or, when the encoding nucleic acid is to be used for eukaryotic expression of the fusion protein, can encode the fusion protein as a plurality of exons with intervening introns.

          The nucleic acids of the present invention  
15 can be in the form of DNA, typically but not invariably double-stranded, or in the form of RNA. Where the DNA encodes the fusion protein as a plurality of exons, the RNA transcript can exist in both unspliced form, containing the introns, and one or more spliced forms.  
20 The nucleic acids of the present invention can also include nonnative nucleotides, alternative internucleotide linkages, or both, typically when the nucleic acids are used as probes rather than for expression of the fusion protein. Probes are useful  
25 for confirming transfection of the nucleic acid into host cells and transcription thereof, although fluorescence emission can often itself be used as a measure of transfection and expression efficiency, as indicated by FIGS. 11 and 13.

30           The nucleic acids of the present invention can be engineered to use codons optimized for expression in various types of host cells, providing nucleic acid molecules of differing nucleic acid

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sequence that encode the same fusion protein. The nucleic acids of the present invention can be in isolated and purified form, e.g. in the form of purified plasmid preparation, and can also be in the form of nucleic acids integrated into one or more chromosomes of a prokaryotic or eukaryotic host cell.

As further demonstrated in the Examples below, the nucleic acids of the present invention can usefully be incorporated into vectors that permit propagation and amplification of the nucleic acids of the present invention in one or more types of eukaryotic and prokaryotic host cells, including mammalian cells, particularly human and rodent cells, insect cells, yeast cells, and bacterial cells, particularly *E. coli* cells, providing nucleic acids for probe construction, further recombinant manipulation, such as mutagenesis, and for cloning into further vectors.

The vectors of the present invention can be plasmid, viral, or combination or variant thereof (e.g., phagemid, cosmid), can be an artificial chromosome (e.g., BAC, YAC, HAC), or can be any other type of cloning and/or expression vector known in the art.

Vectors can be constructed *de novo*, using techniques well known in the art, as are described, *inter alia*, in Sambrook et al., Molecular Cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor Laboratory Press (2000); Ausubel et al. (eds.), Short Protocols in Molecular Biology : A Compendium of Methods from Current Protocols in Molecular Biology, 4th ed., John Wiley & Sons (1999) ; Jones et al. (eds.), Vectors:Cloning Applications: Essential

Techniques (Essential Techniques Series), John Wiley & Son Ltd. (1998); Cid-Arregui et al. (eds.), Viral Vectors: Basic Science and Gene Therapy, Eaton Publishing Company/Bio Techniques Books Division  
5 (2000), the disclosures of which are incorporated herein by reference in their entireties.

Alternatively, the vectors of the present invention can usefully and advantageously be modified from or include relevant portions of vectors presently  
10 used to propagate and/or express DsRed and/or other fluorescent proteins, MHC tetramers, and/or MHC/Ig chimeras.

For example, a variety of DsRed vectors useful as starting material for constructing the  
15 vectors of the present invention are available commercially from Clontech Labs (Palo Alto, CA USA). pDsRed Vector is a prokaryotic expression vector containing the native DsRed sequence flanked by multiple cloning sites; the DsRed coding sequence can  
20 be excised by restriction digest or retrieved by PCR amplification. pDsRed1-1 encodes a DsRed variant having a valine insertion at position 2, with 144 silent changes to optimize expression in mammalian cells. pDsRed1-N1 readily permits mammalian expression  
25 of fusions to DsRed N-terminus; pDsRed1-C1 readily permits mammalian expression of fusions to the DsRed C-terminus.

For example, MHC tetramer expression vectors useful as starting material for constructing the  
30 vectors of the present invention are described in Doherty et al., *Annu. Rev. Immunol.* 18:561-92 (2000); Ogg et al., *Immunol. Lett.* 66(1-3):77-80 (1999); Maini et al., *Immunol. Today* 20(6):262-6 (1999); Doherty,

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*Curr. Opin. Microbiol.* 1(4):419-22 (1998);  
Reichstetter et al., *J. Immunol.* 165(12):6994-8 (2000);  
Kwok et al., *J. Immunol.* 164(8):4244-9 (2000); Liu et  
al., *Proc. Natl. Acad. Sci. USA* 97(26):14596-14601  
5 (2000); Novak et al., *J. Clin. Invest.* 104(12):R63-7  
(1999); Crawford et al., *Immunity* 8:675-682 (1998);  
Kozono et al., *Nature* 369:151-154 (1994); Altman et  
al., *Science* 274:94 (1996); and Altman et al., U.S.  
Patent No. 5,635,363, the disclosures of which are  
10 incorporated herein by reference in their entireties.

For example, vectors for expression of MHC/Ig  
chimeras that are useful as starting material for  
constructing the vectors of the present invention are  
further described in Dal Porto et al., *Proc. Natl.*  
15 *Acad. Sci. USA* 90:6671-6675 (1993); Greten et al.,  
*Proc. Natl. Acad. Sci. USA* 95:7568-7573 (1998); Hamad  
et al., *J. Exp. Med.* 188:1633-1640 (1998); Schneck et  
al., U.S. Pat. Nos. 6,015,884 and 6,140,113, the  
disclosures of all of which are incorporated herein by  
20 reference in their entireties.

Particularly useful among vectors of the  
present invention are those can be used for expressing  
the fusion proteins of the present invention in host  
cells. Details of expression vector construction are  
25 well known in the art and need not be reviewed here.  
In general terms, however, the expression vectors of  
the present invention include those that replicate  
episomally within the host cell and those that are  
integrated into one or more locations of the host cell  
30 genome; expression of the fusion protein from the  
vector can be constitutive or inducible, and the  
expressed fusion protein can be retained within the  
cell or secreted.

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The present invention further includes host cells containing the nucleic acids, typically the vectors, including expression vectors, of the present invention, either episomally or integrated at one or  
5 more locations in the host cell chromosome. Where integrated, the expression vector is usefully present in multiple copies, which increased copy number can be effected by amplification techniques well known in the art (see, e.g., Axel et al., U.S. Patent No. 4,399,216,  
10 the disclosure of which is incorporated herein by reference). Where the host cells include expression vectors of the present invention, the host cells typically further include the fusion proteins of the present invention in one or more cellular compartments.

15 The host cells of the present invention can contain a plurality of vectors, including expression vectors, of the present invention; the plurality of vectors can respectively encode a plurality of different fusion proteins, thus permitting expression  
20 within the cell of heteromeric multimers. As will be further described, such heteromeric multimers can usefully include fusion proteins having identical MHC antigen-presenting moiety and multimerization domains but different GFP-like chromophores.

25 Host cells useful for propagation of the nucleic acids of the present invention include bacterial cells, particularly *E. coli* cells, and eukaryotic cells.

30 Host cells useful for recombinant production of the fusion proteins of the present invention include all types of cells typically used for recombinant expression, including both prokaryotic and eukaryotic

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cells, the latter including human, rodent, insect, and yeast cells.

Insect cells, such as Sf9 and Tni cells, present certain advantages for expression of the fusion proteins of the present invention, including high yield (see, e.g., FIGS. 11 and 13) and likely a low load of endogenous peptide spuriously loaded on the nascent multimers during synthesis. Rodent myeloma cells present certain advantages for expression, particularly where the fusion protein includes most, or all, of an Ig/MHC chimera, fused to a GFP-like chromophore; notable among such advantages is concurrent production of Ig light chain.

Where the fusion protein to be expressed includes an MHC peptide-presenting moiety from a class I MHC molecule, the host cells of the present invention can also usefully and concurrently express  $\beta 2$  microglobulin, either endogenously or through expression of a second recombinant construct.

Where the expressed fusion protein is capable of multimerizing in the intracellular milieu, the host cells typically further include such multimers.

#### Multimeric Complexes

In another aspect, the present invention provides multimeric complexes that include, as plural subunits, fusion proteins of the present invention. When properly complexed with appropriate soluble protein partners (e.g.,  $\beta 2$  microglobulin for fusion proteins having a class I MHC peptide-presenting moiety) and charged with peptide, these intrinsically fluorescent multimeric complexes can be used to label T

lymphocytes based upon their antigen (and MHC) specificity.

In its simplest form, the multimeric complex of the present invention comprises a multimer of the fusion proteins of the present invention, giving the complex a quaternary formula of  $(F)_n$ , where F is a fusion protein as above-described and n is an integer greater than 1.

Multimerization is driven and mediated by the multimerization domains of the fusion protein. Where the multimerization domain(s) of the fusion protein subunits are drawn from DsRed, variants thereof, or related fluorescent proteins, the fusion protein subunits will typically assemble predominantly into tetramers ( $n = 4$ ), with possible formation of weakly associated octamers ( $n = 8$ ). Where the multimerization domain(s) of the fusion protein subunits are drawn instead from *Renilla* GFP or from IgG Fc regions, the fusion protein subunits will typically assemble predominantly into dimers ( $n = 2$ ).

If the fusion protein subunits are expressed in eukaryotic cells, such as rodent myeloma cells or insect cells, the fusion protein subunits will typically self-assemble within the cell into complexes of the expected valency. If the fusion protein subunits are instead expressed in prokaryotic cells, such as *E. coli*, the subunits will typically assemble only after denaturation (e.g., in 8 M urea) and renaturation, as is well known in the MHC tetramer art.

The resulting multimeric complex is intrinsically fluorescent, due to the presence of GFP-like chromophores in each of the participating subunits. The spectral qualities of the complex will

be dictated not only by the spectral properties of the individual GFP-like chromophores present within the complex, but by their collective interaction as well.

A particularly significant interaction among  
5 GFP-like chromophores is fluorescence resonance energy transfer ("FRET").

The multimeric complex of the present  
invention can include fusion protein subunits having  
different GFP-like chromophores, so long as the fusion  
10 protein subunits remain able to multimerize through  
their respective multimerization domains. Typically,  
but not invariably, the fusion protein subunits of such  
heteromultimeric complexes will all have the same MHC  
peptide-presenting moieties. Where the different GFP-  
15 like chromophores have suitable spectral overlap, their  
proximity and relative angular displacement within the  
multimeric complex can permit fluorescence resonance  
energy transfer between the different GFP-like  
chromophores.

20 It is known, for example, that the DsRed  
chromophore can be used as an acceptor in a  
fluorescence resonance energy transfer (FRET) pair with  
enhanced green fluorescent protein (EGFP) or yellow  
fluorescent protein (YFP) mutants. Heikal et al.,  
25 *Proc. Natl. Acad. Sci. USA* 97:11996-12001 (2000).  
Furthermore, fluorescence resonance energy transfer  
among monomers in the native DsRed tetramer has been  
reported. Thus, where the multimeric complex of the  
present invention includes both a fusion protein  
30 subunit having a DsRed GFP-like chromophore and a  
fusion protein subunit having an EGFP GFP-like  
chromophore, the spectral properties of the complex can  
reflect fluorescence resonance energy transfer between

EGFP and DsRed chromophores, allowing the complex to be excited more readily at the EGFP absorption maximum than would have been possible with a homotetramer having only the DsRed chromophores.

5           As noted above, fluorescence resonance energy transfer can occur between and among GFP-like chromophores present in the subunits of the multimeric complexes of the instant invention. FRET can additionally and usefully be established between the  
10 GFP-like chromophore of one or more of the subunits of the complex and a fluorophore associated therewith.

Techniques for associating a fluorophore with a subunit of the multimeric complex, so as to support FRET — by conjugating, linking, or adding the  
15 fluorophore to the subunit, or otherwise modifying a subunit with the fluorophore — are within the knowledge of the skilled artisan.

Depending on the absorption and emission spectra of the fluorophore that is associated with a  
20 particular type of GFP-like chromophore, either the fluorophore or the GFP-like chromophore can serve as the fluorescence energy donor or acceptor.

Fluorophores useful as FRET donors or acceptors when associated with one or more subunits of  
25 the multimeric complex of the present invention include: cyanine 3 (e.g., Cy3, Cy3B); cyanine 5 (e.g., Cy5, Cy5Q); cyanine 5.5; cyanine 7 (e.g., Cy7Q); SYBR® Green (Molecular Probes); fluorescein and fluorescein derivatives (e.g., 6-carboxyfluorescein); rhodamine and  
30 rhodamine derivatives (e.g., dichlororhodamine, dR110, dR6G, dTAMRA, dROX); allophycocyanin and allophycocyanin derivatives (e.g., GT5 APC); R-phycoerythrin; B-phycoerythrin; Y-phycoerythrin;

R-phycoerythrin; C-phycoerythrin; Texas Red®; and proteinaceous fluorophores, e.g., PerCP.

According to a preferred embodiment, the GFP-like chromophore serves as the FRET donor and the fluorophore serves as the FRET acceptor. In this manner, stimulatory light energy of the appropriate wavelength is absorbed by the GFP-like chromophore and is transferred intramolecularly to the fluorophore, which then fluorescently emits the energy at a different, longer wavelength.

This arrangement can usefully accelerate the rate of fluorescent light emission.

The rate of fluorescent decay of some GFP-like fluorophores, after light stimulation, is relatively slow. For example, the energized DsRed chromophore decays to its ground state by fluorescent emission over a time period of microseconds. In contrast, intramolecular energy transfer by FRET between a GFP-like chromophore, such as DsRed, and an associated fluorophore typically occurs in nanoseconds. As a result, fluorescent emission by the fluorophore may be faster than fluorescent emission by the GFP-like fluorophore. The effect of this difference in the rate of fluorescent energy emission is that within a brief, predetermined time period, fluorescent light emission by the fluorophore, coupled by FRET to the GFP-like chromophore is greater, and the signal appears brighter. In contrast, within the same time period, fluorescent light emission by the GFP-like chromophore, unassisted by FRET and a fluorophore is lower, and the signal appears dimmer.

Increasing the rate of fluorescent emission of GFP-like chromophores, such as by the FRET-mediated

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mechanism described above, can be particularly advantageous when using flow cytometry. FRET-mediated acceleration of fluorescence emission by associating a fluorophore with a GFP-like chromophore will also be desirable in any other application in which signal acquisition must be made quickly or signal maximized.

In general, suitable selection of GFP-like chromophores to be included in the fusion protein subunits multimerized in the complex (and optionally FRET donors and acceptors conjugated thereto) will allow fine tuning of the spectral characteristics of the complex. One important utility of such tuning is to match the absorption and emission characteristics of the complex to the excitation and detection parameters of the analytical instrument, typically a flow cytometer. Conversely, as is further discussed below, lasers, filter sets, and detectors can be chosen that match the absorption and emission characteristics of the multimeric complex.

For detectable labeling of antigen-specific T lymphocytes, the multimeric complex of the present invention will further include soluble protein partners and peptides. The above-described  $(F)_n$  complexes are useful intermediates, however, in the preparation of these labeling reagents, and will usefully be sold to end users desiring themselves to complete formation of the complex.

Thus, in a further aspect, the invention includes intrinsically fluorescent, multimeric protein complexes comprising a plurality of subunits, the subunits having the quaternary formula in the complex of  $(F_1S_1)_n$ , where F, as above described, is a recombinant fusion protein of the present invention, S

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is a soluble protein, and  $n$  is an integer greater than 1.

S is selected from the group consisting of  $\beta 2$  microglobulin, class II  $\beta$  MHC peptide-presenting soluble derivatives, and class II  $\alpha$  MHC peptide-presenting soluble derivatives: S is  $\beta 2$  microglobulin when F includes a class I  $\alpha$  MHC peptide-presenting moiety; S is a class II  $\beta$  MHC peptide-presenting soluble derivative when F includes a class II  $\alpha$  MHC peptide-presenting moiety; and S is a class II  $\alpha$  MHC peptide-presenting soluble derivative when F includes a class II  $\beta$  MHC peptide-presenting moiety.

Typically, the soluble protein is derived from the same taxonomic species as provides the MHC peptide-presenting moiety of the fusion protein in the complex. For example, when the fusion protein includes MHC class I  $\alpha$  domains from mouse, the soluble  $\beta 2$  microglobulin used in the multimeric complex will be murine  $\beta 2$  microglobulin. For example, where the fusion protein includes human HLA-DQ $\alpha$  domains, the MHC peptide-presenting soluble derivative included within the complex will include human class II  $\beta$  domains, such as HLA-DQ $\beta$  domains.

The soluble protein can be coexpressed with the fusion protein subunits in a single host cell, facilitating self-assembly of the  $(F_1S_1)_n$  complex within the cell, or can be added to a prior-assembled complex having the quaternary formula  $F_n$ . When the fusion protein subunits of the multimeric complex include a class II MHC peptide-presenting moiety, the class II MHC peptide-presenting soluble derivative will most typically be coexpressed therewith, particularly when expression of the fusion protein is effected in

eukaryotic cells; when the fusion protein subunits of the multimeric complex include a class I  $\alpha$  MHC peptide-presenting moiety, the  $\beta 2$  microglobulin soluble protein can readily be coexpressed therewith or added after  
5 assembly of the  $(F)_n$  multimer. Coexpression of  $\beta 2$  microglobulin in eukaryotic cells can result either from expression from a recombinant construct or, depending upon the choice of host cell, from endogenous expression by the host cell itself.

10           The multimeric complex, properly assembled with soluble protein, can thereafter be charged with specific peptide, providing an intrinsically fluorescent reagent that is useful for detectable labeling (and, in other methods, activation) of T  
15 lymphocytes based upon their antigen specificity.

          It would be understood that the complex above-described - that is, inclusive of fusion protein and soluble protein subunits but lacking peptide antigen - can be charged with an enormous  
20 variety of peptide antigens, each peptide conferring its own specificity on the completed labeling reagent. The above-described  $(F_1S_1)_n$  complex is thus extremely useful in the preparation of labeling reagents; and often, the intrinsically fluorescent complexes of the  
25 present invention will usefully be sold to the end-user in such form to permit the end-user to charge the complex with the peptide antigen of his or her choice.

          Charged with peptide antigen, the multimeric complex is directly usable to label T lymphocytes based  
30 upon the antigen- (and MHC-) specificity of their T cell receptors. Thus, in another aspect, the invention provides an intrinsically fluorescent, multimeric protein complex for labeling T lymphocytes, the complex



comprising a plurality of subunits, the subunits having the quaternary formula in the complex of  $(F_1S_1P_1)_n$ , where F, as above, is a recombinant fusion protein of the present invention, S is a soluble protein as above-  
5 described, P is a peptide antigen, and n is an integer greater than 1.

The peptide antigen will be bound, as in MHC tetramers and MHC/Ig chimeras, by the MHC peptide-presenting domains of the multimeric complex of the  
10 present invention: in a class I MHC intrinsically fluorescent multimer, by the  $\alpha 1$  and  $\alpha 2$  domains of the class I  $\alpha$  MHC peptide presenting moiety of the fusion protein subunit; in a class II multimer, by the  $\alpha 1$  and  $\beta 1$  domains of the MHC peptide-presenting moiety and  
15 soluble derivatives of the complex.

Where the complex of the present invention is a class I multimer, the peptide antigen will typically be no fewer than about 5, more typically no fewer than about 6, more typically no fewer than about 7, often no  
20 fewer than about 8 or 9 amino acids in length, and will typically be no more than about 15, typically no more than about 14, more typically no more than about 13, 12, or 11, and often no more than about 10, or 9 amino acids in length.

25 As is well known in the immunologic arts, the antigenic peptides can have a wider length range when complexed to MHC class II. Thus, for charging class II multimers, the peptides will typically be no fewer than about 8, typically no fewer than about 9, 10, 11 or 12  
30 amino acids in length, and will typically be no more than about 30, typically no more than about 25, typically no more than about 19, 18, 17, or 16 amino acids in length, often no more than about 12 amino

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acids in length. And as noted above, for class II  
multimers the peptide can be covalently fused to the N  
terminus of the MHC peptide-presenting moiety or  
soluble derivative, Kozono *et al.*, *Nature* 369:151-154  
5 (1994); Liu *et al.*, *Proc. Natl. Acad. Sci. USA*  
97:14596-14601 (2000), rather than noncovalently  
associated

Peptides will typically be substantially pure  
and of uniform sequence, such as can advantageously be  
10 prepared by chemical synthesis optionally followed by  
further purification, as, e.g., by HPLC. However, for  
some uses, a collection of peptides - e.g., a  
collection of peptides useful for vaccinating against  
CMV, as is described in WO 00/75180, the disclosure of  
15 which is incorporated herein by reference in its  
entirety - can be used to charge the intrinsically  
fluorescent multimers of the present invention. In  
this latter case, a population of multimers is created  
that will collectively label T lymphocytes that  
20 recognize any of the charging peptides. For such  
purposes, the peptides can be advantageously be created  
by endoproteolytic digest of a native protein, such as  
a viral protein.

Peptides can include nonnative amino acids  
25 and nonnative linkages, where such nonnative amino  
acids and/or nonnative linkages do not interfere with  
the ability of the intrinsically fluorescent complex to  
label T lymphocytes. For example, nonnative amino  
acids and/or linkages can be used to assess the T cell  
30 response to immunization with a protein or peptide that  
includes such nonnative amino acids or linkages, or to  
detect T lymphocytes specific for the peptide in its  
native form where the nonnative amino acids and/or

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linkages do not affect the three dimensional structure of the peptide.

Where the multimeric complexes of the present invention are prepared by recombinant expression in eukaryotic cells, particularly mammalian cells, the multimer may spuriously include peptides endogenous to the host cell. Although expression in insect cells can decrease the amount of endogenous peptide loading, a certain percentage of peptide binding sites can nonetheless be occupied prior to intentional loading. In such cases, peptide charging of the multimeric complexes will include some degree of peptide exchange. Exchange can be facilitated at higher temperatures, such as 37°C, and by using higher concentrations of charging peptide. Where the concentration of peptide used for charging is high, and indeed at any peptide concentration, dialysis can optionally be performed after charging is completed to reduce the concentration of unbound peptide.

The sequence of the charging peptide will be dictated by the use to which the labeling reagent is to be put. For example, to detect the antigen-specific CD8<sup>+</sup> T cell response to viral infection or viral vaccination, a peptide sequence derived from viral protein will be used to charge a class I intrinsically fluorescent multimer; to detect the presence of autoimmune T lymphocytes associated with multiple sclerosis, a peptide derived from myelin basic protein can be used to charge a class I or class II intrinsically fluorescent multimer.

As noted, particularly useful are peptides from viruses, such as HIV (human immunodeficiency virus), SIV (simian immunodeficiency virus), CMV

(cytomegalovirus), HBV (hepatitis B virus), HCV (hepatitis C virus), HSV (herpes simplex virus), EBV (Epstein Barr virus), and HPV (human papilloma virus). Also useful are bacterial peptides, such as peptides  
5 from mycobacteria, including TB. Another class of peptides advantageously used are peptides derived from cancer-related antigens, particularly cancer antigens used in potential cancer vaccines, notably vaccines against melanoma, prostate cancer, and breast cancer.

10           Peptides used in the multimeric complexes of the present invention can also be selected on the basis of their mimicry of non-peptide structures, such as carbohydrates, Luo et al., *J. Biol. Chem.* 275(21):16146-54 (2000); O et al., *Biochem. Biophys.*  
15 *Res. Commun.* 5;268(1):106-11 (2000); Grothaus et al., *Vaccine* 18(13):1253-63 (2000); and Phalipon et al., *Eur. J. Immunol.* 27(10):2620-5 (1997).

          The multimeric complexes of the present invention, with or without associated peptide (i.e.,  
20 with either of quaternary formulas  $(F_1S_1)_n$  or  $(F_1S_1P_1)_n$ ), can usefully be provided as an aqueous composition, typically including buffers, salts and/or stabilizers, e.g., phosphate buffered saline with gelatin and 0.1% sodium azide, and it is thus another aspect of the  
25 present invention to provide such compositions. Alternatively, the complexes can be provided as nonaqueous, lyophilized compositions.

#### Methods of Use

          Once charged with a given peptide antigen,  
30 the intrinsically fluorescent complexes of the present invention can bind to T lymphocytes having antigen

receptors that are specific for the given peptide and MHC peptide-presenting domains of the multimeric complex. Because the peptide antigens and MHC peptide-presenting domains of the complexes of the present invention can both be varied, the intrinsically fluorescent multimeric complexes of the present invention can be used, in general, fluorescently to label a near infinite variety of T lymphocytes based upon the antigen (and MHC) specificity of their antigen receptors.

It is, therefore, another aspect of the present invention to provide methods for using the multimeric complexes of the present invention detectably to label (stain) T lymphocytes based upon the specificity of their antigen receptors.

In a first embodiment, the method comprises contacting a T lymphocyte to be labeled with an intrinsically fluorescent multimeric complex of the present invention, the complex having peptide antigen and MHC peptide-presenting domains for which the antigen receptor of the T lymphocyte is specific, for a time and under conditions sufficient to permit detectable binding of the complex to the T lymphocyte.

In the present context, a T lymphocyte is said to be specific for a peptide antigen when the affinity of its antigen receptor (TCR) for the peptide antigen in the MHC context of the complex is sufficiently high as to confer upon the complex as a whole avidity for the T lymphocyte that is sufficient to achieve detectable binding of the complex to TCRs on the lymphocyte surface.

Often, the peptide antigen(s) for which the T lymphocyte is said to be specific by this definition

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will also be capable of stimulating cytokine expression by the T lymphocyte; such a functional response is not, however, required, since the utility of the multimeric complexes of the present invention, as for MHC tetramers, is not limited to labeling and identification of functionally responsive T lymphocytes. Furthermore, by this definition it is not impossible for a single T lymphocyte, having a single  $\alpha\beta$  or  $\gamma\delta$  TCR species on its surface, to be said to be specific for a plurality of (typically closely related) peptide antigens. In such cases, the TCR will typically have greater affinity for one of the peptides than for the others.

Conditions and times adequate for such labeling can conveniently be adapted from those used in the art for staining T lymphocytes with MHC tetramers or MHC/Ig fusions.

For example, Altman et al., *Science* 274:94-96 (1996) stain 200,000 cytotoxic lymphocytes with MHC tetramers by incubation at 4°C for one hour at a concentration of tetramer of approximately 0.5 mg/ml; the NIAID Tetramer facility (<http://www.niaid.nih.gov/reposit/tetramer/genguide.htm>) presently recommends staining at each of 4°C, room temperature, and 37°, for 15-60 minutes, to optimize signal to noise ratio, with decreasing incubation times used for higher temperatures; Greten et al., *Proc. Natl. Acad. Sci. USA* 95:7568-7573 (1998) stain  $1 \times 10^6$  peripheral blood mononuclear cells at 4° with 3  $\mu$ g of MHC class I MHC/Ig chimera.

Thus, T lymphocytes can conveniently be labeled with the intrinsically fluorescent multimeric complexes in the methods of the present invention using

at least about 0.1  $\mu$ g, typically at least about 0.25  $\mu$ g, more typically at least about 1  $\mu$ g, 2  $\mu$ g, 3  $\mu$ g, 4  $\mu$ g, or even at least about 5  $\mu$ g of multimer to label about  $10^4$ ,  $10^5$ ,  $10^6$  or even  $10^7$  peripheral blood mononuclear cells using an incubation of 15 - 60 minutes at a temperature between 4°C and 37°C.

Starting with these broad, exemplary, guidelines, those skilled in labeling T lymphocytes using MHC tetramers, MHC/Ig fusions, and fluorophore-conjugated antibodies will readily be able to determine optimal labeling conditions. Variables that affect the amount of multimeric reagent to be used and the temperature and duration of staining include those related to the T lymphocytes - the number of T lymphocytes in the sample that are specific for the peptide antigen (and MHC) of the complex, the total number of cells in the sample, the form of the cellular sample (e.g., whole blood, whole blood after red blood cell lysis, Ficoll-purified peripheral blood mononuclear cell (PBMC) fraction) - and those related to the multimeric complex, including the stoichiometry and molecular weight of the labeling complex, the identity of the peptide antigen, and the choice of MHC alleles included in the complex.

To optimize labeling conditions, labeling reactions can readily be performed using parallel aliquots of the cellular sample to be labeled using a single species of multimeric complex and varying labeling conditions (e.g., temperature, duration, complex concentration, cell number, cell concentration, cellular purity). Negative controls can include labeling reactions using no multimer, using multimer lacking peptide, and/or using multimer containing MHC

peptide-presenting domains and/or peptide antigen that will not be recognized by T lymphocytes in the cellular sample. Effectiveness of labeling can be readily determined for each aliquot by flow cytometric enumeration of T lymphocytes in the sample that bind the fluorescent complex. As is well known in the flow cytometric arts, the labeled cells can be washed prior to flow cytometry to remove unbound complex from the cells and medium. All of these optimizing techniques and approaches are routine, and routinely performed by technicians, in the flow cytometric arts.

Typically, the T lymphocytes to be labeled are present within a heterogeneous sample of cells, and the goal of labeling is to detect, and often to enumerate, the antigen specific T lymphocytes within this population.

Thus, in another aspect, the present invention provides methods for detecting, in a sample of cells, T lymphocytes that are specific for a chosen antigen. The method comprises contacting the sample with an intrinsically fluorescent multimeric complex according to the present invention, wherein the peptide antigen of the complex is the chosen antigen and the MHC presenting domains of the complex are those for which the T lymphocytes desired to be detected will be restricted, for a time and under conditions sufficient to permit detectable binding of the complex to T lymphocytes in the sample that are specific for the chosen antigen and MHC; and then detecting specific T lymphocytes in the sample by the fluorescence of the complex bound thereto.

The detection of cell-bound fluorescence is typically performed using a flow cytometer, such as a



FACSVantage™, FACSVantage™ SE, or FACSCalibur™ flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). The lasers chosen for excitation will be determined by the absorption spectrum of the multimeric complex and of any additional fluorophores desired to be detected concurrently in the sample. For example, if the multimeric complex has the spectral characteristics of native DsRed – e.g., a homotetramer in which the fusion protein subunits all have the native DsRed GFP-like chromophore – a standard argon ion laser with 488 nm line can be used for excitation. For detection, the filter sets and detector types will be chosen according to the emission spectrum of the multimeric complex and of any additional fluorophores desired to be detected in the sample. For example, if the multimeric complex has the spectral characteristics of native DsRed, with emission maximum at about 583 nm, fluorescence emission from the complex can be detected in the FL2 channel using a PE setup.

Alternatively, cell-bound complex fluorescence can be detected using a microvolume fluorimeter, such as the IMAGN 2000 (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Applications of microvolume fluorimetry to, and conditions for, characterization of blood cell are described, *inter alia*, in Seghatchian et al., *Transfus. Sci.* 22(1-2):77-9 (2000); Glencross et al., *Clin. Lab. Haematol.* 21(6):391-5 (1999); and Read et al., *J. Hematother.* 6(4):291-301 (1997).

Alternatively, cell-bound complex fluorescence can be detected using a laser scanning cytometer (Compucyte Corp., Cambridge, MA, USA).

Cell-bound fluorescence of the multimeric complex can also be detected directly on a microscope slide, using conditions essentially as described in Skinner et al., "Cutting edge: In situ tetramer  
5 staining of antigen-specific T cells in tissues," *J. Immunol.* 165(2):613-7 (2000).

The T lymphocyte-containing sample can be a whole blood sample, typically a peripheral venous blood specimen drawn directly into an anticoagulant  
10 collection tube (e.g., EDTA-containing or heparin-containing Vacutainer™ tube, Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA).

Advantageously, the T lymphocyte-containing sample can also be a whole blood sample that has been  
15 treated before detection with a red blood cell (RBC) lysing agent as is described, *inter alia*, in Chang et al., U.S. Patent Nos. 4,902,613 and 4,654,312; lysing agents are well known in the art and are available commercially from a number of vendors (FACS™ Lysing  
20 Solution, Becton Dickinson Immunocytometry Systems, San Jose, CA, USA; Cal-Lyse™ Lysing Solution, Caltag Labs, Burlingame, CA, USA; No-Wash Lysing Solution, Beckman Coulter, Inc., Fullerton, CA). The sample can optionally be washed after RBC lysis and before  
25 detection.

The sample within which T lymphocytes are desired to be detected according to the methods of the present invention can also be a peripheral blood fraction, advantageously a mononuclear cell (PBMC)  
30 fraction. PBMCs can be prepared according to any of the well-known art-accepted techniques, among which are centrifugation through a density medium, such as Ficoll-Paque (Amersham Pharmacia Biotech, Piscataway,

2015-01-09 10:45:49

NJ, USA) and centrifugation directly in a specially designed cell preparation blood collection tube (e.g., Vacutainer™ CPT™ Cell Preparation Tube, Becton Dickinson, Franklin Lakes, NJ, USA).

5           The sample within which T lymphocytes are desired to be detected according to the methods of the present invention can also advantageously be a sample enriched in T lymphocytes. For example, the sample can be a sample of cultured lymphocytes (as from a clonal  
10 cell line or multiclonal culture), lymphocytes extracted or eluted from a tissue having lymphocytic infiltrate (e.g., tumor infiltrating lymphocytes extracted from a tumor biopsy and optionally expanded in culture), lymphocytes drawn from lymphatics or  
15 thymus, or lymphocytes obtained after at least a first round of fluorescence-activated cell sorting.

          In another embodiment, the method further comprises enumerating the antigen-specific T lymphocytes so detected. Enumeration can conveniently  
20 be expressed in the form of a total cell count, percentage of antigen-specific T lymphocytes among cells assayed (either total, mononuclear, or T lymphocytic), or percentage of antigen-specific lymphocytes in a T cell subset.

25           For several of these metrics, it is necessary additionally to quantitate the total number of T lymphocytes within the sample as a whole, or the total number of T lymphocytes of a particular subset within the sample as a whole.

30           Thus, in other embodiments, the method of the present invention further comprises contacting the sample with at least one fluorophore-conjugated antibody, the antibody selected from the group

2017-03-10 10:04:49

consisting of pan-T antibodies and T cell subsetting antibodies, and then detecting fluorescence concurrently from the multimeric fluorescent complex and from the fluorophore-conjugated antibodies. By

5 "pan-T antibody" is intended an antibody that recognizes a surface marker or epitope present on all, or substantially all, T lymphocytes. By "T cell subsetting antibody" is meant an antibody that binds to a surface marker present on fewer than all T  
10 lymphocytes.

Antibodies usefully used in this embodiment include antibodies specific for CD3, CD4, CD8, CD45RO, CD45RA, and CD27. As would be understood, the antibodies would typically be specific for the marker  
15 as expressed by the taxonomic species (human, mouse, rat, etc.) whose T lymphocytes are being detected, or would be cross-reactive therewith.

As is well known in the flow cytometric arts, fluorescence emission from the pan-T and/or T  
20 lymphocyte subsetting antibodies can be used for any or all of triggering data acquisition, live gating, or gating prior-acquired data.

It is often advantageous in the methods of the present invention to acquire a large number of  
25 events since, in many samples, antigen-specific T lymphocytes occur infrequently. In addition, it is possible at times to improve the signal to noise ratio for detecting such rare antigen-specific T cell events by triggering or gating on fluorescence from antibodies  
30 specific for T lymphocyte activation antigens.

Thus, in another embodiment, the method further comprises contacting the sample with at least one fluorophore-conjugated antibody specific for a T

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cell activation antigen, and then detecting  
fluorescence concurrently from the multimeric  
fluorescent complex and from the fluorophore-conjugated  
antibodies. The antibodies can usefully be specific  
5 for an activation antigen selected from the group  
consisting of CD69, CD25, CD71 and MHC class II (for  
labeling human T lymphocytes, advantageously HLA-DR).

Advantageously, the antibodies used in these  
embodiments of the methods of the present invention  
10 will be prior-conjugated directly to a fluorophore,  
typically a fluorophore whose emission is flow  
cytometrically distinguishable from that of the  
intrinsically fluorescent multimeric T cell labeling  
complex and from that of other fluorophores  
15 concurrently used in the method. Fluorophores can  
usefully be selected from the group consisting of  
fluorescein isothiocyanate (FITC), phycoerythrin (PE),  
peridinin chlorophyll protein (PerCP), allophycocyanin  
(APC), Texas Red, Alexa Fluor 488 (Molecular Probes,  
20 Inc., Eugene Or.), and the tandem fluorophores PerCP-  
Cy5.5, PE-Cy5, PE-Cy7, and APC-Cy7. Antibodies can  
also usefully be conjugated to biotin, permitting  
second stage detection using fluorophore-labeled  
streptavidin.

25 The methods of the present invention for  
detecting and enumerating antigen-specific T  
lymphocytes can be used for the same purposes as are  
prior art methods for detecting and enumerating  
antigen-specific T lymphocytes, including methods based  
30 upon surface labeling of TCR (use of MHC tetramers and  
MHC/Ig chimeras), as well as methods based upon  
detecting functional responses of antigen-specific T  
cells (limiting dilution assay, enzyme-linked

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immunospot assay, and flow cytometric detection of  
intracellular cytokine expression, Waldrop et al., *J.*  
*Clin. Invest.* 99(7):1739-50 (1997)). The methods can  
thus be used, e.g., to assess CD4<sup>+</sup> and CD8<sup>+</sup> T cell  
5 responses to infection, to vaccines, and in  
autoimmunity.

Depending upon the instrument used, detection  
of antigen-specific T lymphocytes can be coupled  
directly or indirectly to their sorting, thus  
10 providing, in other aspects of the invention, methods  
for enriching a sample in, and for depleting a sample  
of, T lymphocytes that are specific for a chosen  
antigen.

In general, these methods comprise contacting  
15 the sample with an intrinsically fluorescent multimeric  
complex of the present invention, wherein the peptide  
antigen of the complex is the chosen antigen and the  
MHC presenting domains of the complex are those for  
which the T lymphocytes desired to be enriched or  
20 depleted will be restricted, for a time and under  
conditions sufficient to permit detectable binding of  
the complex to T lymphocytes in the sample that are  
specific for the chosen antigen and MHC. After  
binding, labeled T lymphocytes are enriched or depleted  
25 based upon the fluorescence of the complex bound  
thereto.

Such methods are conveniently performed using  
a fluorescence activated cell sorter: sorting based at  
least in part upon fluorescence from the multimeric  
30 complex of the present invention directly depletes the  
sample from which the cells are removed and enriches  
the aliquot into which the cells are placed.

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It is possible, however, to use the multimers of the present invention to enrich or deplete samples in T lymphocytes of particular antigen specificity using approaches other than fluorescence-activated cell  
5 sorting.

For example, T lymphocytes stained specifically with the multimer can be separated magnetically, rather than fluorimetrically, by further conjugation of the TCR-bound complex to a  
10 superparamagnetic particle. This can be done, e.g., using an antibody that is conjugated to a magnetic particle and that is specific for an epitope of the fusion protein (e.g., where DsRed contributes GFP-like chromophore and/or multimerization domains, the  
15 antibody can be the DsRed-specific antibody available commercially from Clontech Labs, Palo Alto, CA, USA).

As another example, T lymphocytes stained specifically with the multimer can be separated using biotin/avidin affinity interactions, rather than  
20 fluorescence, by further conjugation of the TCR-bound complex to biotin, followed by use of an avidin affinity matrix. This further labeling of the multimeric complex can be done indirectly using a biotin-conjugated antibody specific for an epitope of  
25 the fusion protein. Alternatively, the multimer can itself be prior-conjugated to biotin, either chemically or, upon engineering of a BirA substrate peptide into the complex (typically the fusion protein), enzymatically.

30 Samples enriched in antigen-specific T cells according to the methods of the present invention can be used *in vitro* for study of specific interactions of antigen-specific T cells with antigen-presenting cells,

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cytotoxic targets, B cells, or other cellular elements of the immune system. Samples enriched in antigen-specific T cells according to the method of the present invention can also be used *in vitro* to modify such  
5 interactions. See, e.g., Dal Porto *et al.*, *et al.*, *Proc. Natl. Acad. Sci. USA* 90:6671-6675 (1993).

Samples enriched in antigen-specific T cells according to the methods of the present invention can also be used for *in vivo* therapeutic intervention, such  
10 as for tumor immunotherapy. See, e.g., Oelke *et al.*, *Clin. Cancer Res.* 6(5):1997-2005 (2000). Conversely, samples depleted in particular antigen-specific T cells according to the methods of the present invention can be advantageously administered to patients where  
15 infusion of blood fractions containing T cells having that antigen specificity would occasion disease or morbidity.

As an alternative utility to fluorescent detection of peptide specific T-cells, the multimeric  
20 protein complexes of the instant invention can also advantageously be used to stimulate peptide antigen-specific, MHC-dependent, activation of T cells.

As is known in the art, complexes comprising multiple MHC molecules loaded with peptide are more  
25 potent than non-complexed, monomeric MHC-peptide combinations for stimulating T-cells that specifically bind the peptide by a MHC dependent mechanism. See, for example, Stryhn A., *et al.*, "Preformed purified peptide/major histocompatibility class I complexes are  
30 potent stimulators of class I-restricted T cell hybridomas," *Eur. J. Immunol.*, 24(6):1404-9 (June 1994); Cochran J.R., *et al.*, "The relationship of MHC-peptide binding and T cell activation probed using

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chemically defined MHC class II oligomers," *Immunity*,  
12(3):241-50 (March 2000); and Piccirillo C.A., Shevach  
E.M., "Cutting edge: control of CD8+ T cell activation  
by CD4+CD25+ immunoregulatory cells," *J. Immunol.*,  
5 167(3):1137-40 (August 2001), the disclosure of each of  
which is incorporated herein by reference in its  
entirety.

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Tetramers of MHC peptide-presenting moieties,  
formed by interaction of the multimerization domains  
10 contained within the DsRed subunits of the multimeric  
complexes of the instant invention, can therefore be  
loaded with peptide and incubated with a mixed  
population of T-cells. Subpopulations of T-cells  
bearing T-cell receptors capable of specifically  
15 binding the peptide in a MHC-dependent manner will be  
activated. The greater potency of the tetramers, as  
compared to monomers, dimers or trimers, facilitates  
activation of T-cells using lower concentration of  
peptide-MHC complexes. This lowers cost of reagents  
20 and decreases spurious activation caused by high  
concentrations of activating peptide-MHC complexes.  
Alternatively, the greater potency of tetramers  
facilitates activation of T-cell subpopulations bearing  
receptors with lower affinity for the peptide.

25 According to an alternative embodiment,  
clonal populations of T-cells with defined peptide  
specificity can all be activated using lower  
concentrations of tetrameric peptide-MHC complexes.

Thus, in another aspect, the present  
30 invention provides a method of activating peptide  
antigen-specific, MHC-dependent, T-cells, comprising  
contacting, for time sufficient to effect activation, a  
T-cell with an intrinsically fluorescent, self-

multimerizing multimeric complex of the instant invention to which the peptide has been bound.

### Kits

The multimeric complex compositions of the present invention can usefully be provided in the form of kits that facilitate the practice of the methods of the present invention. Thus, in another aspect, the present invention provides kits comprising the intrinsically fluorescent multimeric complexes of the present invention.

In one embodiment, the kits of the present invention include, as separate compositions, an intrinsically fluorescent  $(F_1S_1)_n$  multimer of the present invention, and an antigenic peptide. As noted above, the choice of peptide will depend upon the specificity desired for the labeling reagent.

In some embodiments, a further composition, comprising a second, negative control, peptide is usefully included. The negative control peptide, when used to charge the intrinsically fluorescent  $(F_1S_1)_n$  multimer of the kit, will provide a multimeric complex that has been prior shown not to label a significant percentage of T lymphocytes in the sample to be tested, thus providing a measure of the background attributable to nonspecific binding of the multimer to cells in the sample.

In other embodiments, the kit will further include at least one pan-T or T lymphocyte subsetting antibody. As noted above, by "pan-T antibody" is intended an antibody that recognizes a surface marker or epitope present on all, or substantially all, T

lymphocytes. By "T cell subsetting antibody" is intended an antibody that binds to a surface marker present on fewer than all T lymphocytes.

Where the intrinsically fluorescent multimer is prior-charged with peptide, such kits typically will include, as separate compositions, a multimer and at least one pan-T or subsetting antibody; where the intrinsically fluorescent multimer is provided without prior-charging with peptide (i.e., as an  $(F_1S_1)_n$  structure), the kit will usefully include as separate compositions an intrinsically fluorescent multimer, a peptide, and at least one pan-T or subsetting antibody.

Pan-T and subsetting antibodies usefully included in such kits include those specific for CD3, CD4, CD8, CD45RO, CD45RA, and CD27. As would be understood, the antibodies would typically be specific for the surface marker as expressed by T lymphocytes of the taxonomic species whose T lymphocytes are recognized by the intrinsically-fluorescent multimeric complex (e.g., anti-murine CD3 antibody in a kit that includes a multimeric complex having murine MHC peptide-presenting moieties and soluble derivatives).

In other embodiments, the kit will include at least one antibody to a T cell activation antigen. T cell activation antigens usefully included in such kits include those specific for CD69, CD25, CD71 and, for labeling human T lymphocytes, HLA-DR. Such kits can advantageously also include a pan-T and/or T cell subsetting antibody as above-described.

In kits that include antibodies, the antibodies are usefully provided prior-conjugated directly to a fluorophore, typically a fluorophore that is flow cytometrically distinguishable from the

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emission of the intrinsically fluorescent multimeric T cell labeling complex and from the emission of other fluorophores used in the kit. Fluorophores can usefully be selected from the group consisting of  
5 fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), allophycocyanin (APC), Texas Red, Alexa Fluor 488 (Molecular Probes, Inc., Eugene Or.), and the tandem fluorophores PerCP-Cy5.5, PE-Cy5, PE-Cy7, and APC-Cy7. Antibodies in kits  
10 of the present invention can also usefully be conjugated to biotin, permitting second stage detection using labeled streptavidin.

Also usefully included in kit embodiments that include antibodies are isotype control antibodies;  
15 as is well known, such isotype control antibodies are antibodies of identical isotype to the fluorophore-conjugated antibodies but directed to irrelevant specificities, such as KLH, suitable to provide measurements of background binding and fluorescence.

20 Other kit embodiments further include a red blood cell lysing agent, as is described, *inter alia*, in Chang et al., U.S. Patent Nos. 4,902,613 and 4,654,312; lysing agents are well known in the art and are available commercially from a number of vendors  
25 (FACS Lysing Solution, Becton Dickinson Biosciences Immunocytometry Systems, San Jose, CA, USA; Cal-Lyse<sup>TM</sup> Lysing Solution, Caltag Labs, Burlingame, CA, USA; No-Wash Lysing Solution, Beckman Coulter, Inc., Fullerton, CA).

30 Many of the kit embodiments will further include instructions for labeling (staining) with the multimeric complex and for performing flow cytometric analysis.

Other kit embodiments further include an antibody capable of specifically recognizing and binding DsRed, or other GFP-like chromophores, which antibody is conjugated to a fluorophore. In this manner, indirect immunofluorescence techniques can be employed. For example, in a first step, the intrinsically fluorescent, multimeric protein complex of the instant invention bearing peptide is incubated with T-cells to effect specific binding of peptide to T-cell receptors. Thereafter, in a second step, the fluorescently labeled secondary antibody is incubated with the T-cells such that the antibody binds the GFP-like chromophore bound to T-cells. For analysis, the fluorophore associated with the secondary antibody is stimulated with light of the appropriate wavelength, which typically, but not invariably, is chosen so as to avoid substantial stimulation of fluorescent emission by the GFP-like fluorophore.

Indirect immunofluorescence can also be accomplished in three steps. As for the two step method, a second antibody is used to bind the GFP-like chromophore; the second antibody, however, is not itself conjugated to a fluorophore. Rather, in a third step, a fluorescently labeled third antibody is added which is specific for, and binds to, the second antibody. During analysis, the fluorophore conjugated to the third antibody is stimulated by light of the appropriate wavelength. Thus, kits of the instant invention may further include a second antibody that recognizes the GFP-like chromophore and a fluorescently labeled third antibody that recognizes the second antibody.

As used herein, the term "antibody" encompasses whole antibodies, including, but not limited to, those of the IgG, IgM, IgA, IgE, and IgD classes; and antigen-specific antibody fragments, including, but not limited to, Fab, (Fab')<sub>2</sub>, phage-displayed antibodies (phAb), and single-chain variable region antibodies (scFv).

The following examples are provided by way of illustration and not by way of limitation.

10

EXAMPLE 1

Construction of an expression vector for the expression of a MHC-DsRed fusion protein.

Using standard molecular biology techniques, a DNA sequence encoding the extracellular domain (ECD) of murine H2-Ld MHC class I protein is removed from the H2-Ld cDNA and ligated into the Multiple Cloning Site of the vector pDsRed2-N1 (BD Biosciences-Clontech Cat.#6973-1) digested with the enzymes HindIII and BamHI. This results in an in-frame fusion of the coding sequence of the ECD, oriented at the amino-terminal part of the fusion, with the coding sequence of the DsRed chromophore, oriented at the carboxy-terminal part of the fusion. Construction of the fusion construct is shown schematically in FIG. 1.

The H2Ld-DsRed2 fusion construct is then ligated into the NdeI and NotI restriction enzyme sites of pBD1031S1, a BD Biosciences-PharMingen vector developed from PVL1393, Cat. #21201P. The pBD1031S1 vector is a dual expression vector containing a T7 RNA polymerase promoter for expression of the fusion construct in bacteria and a baculovirus polyhedrin

promoter for expression of the fusion construct in a variety of insect cell lines, including Sf9 and Tni cells. A schematic and restriction enzyme map of the expression vector, called dSS-H2LdECD-DsRed-pBD1031S1, is shown in FIG. 2.

After completion of the ligation reaction, using standard techniques, the ligated DNA is purified and used to transform E. coli. bacteria that are then grown overnight on agar plates containing ampicillin to select for transformed cells. Individual amp resistant colonies are then grown in liquid culture; aliquots of bacteria are withdrawn, from which plasmid DNA is isolated and purified.

Plasmid DNA is then doubly digested with NdeI and NotI restriction enzymes to test for the presence in the vector of the Ld-DsRed insert. The digests are separated by electrophoresis in 0.7% agarose gel that is then stained with ethidium bromide, or other fluorescent DNA dye, illuminated with UV light and photographed using a video still camera. Plasmids containing the expected 1.5kb insert are reserved for further analysis by sequencing (see below) to confirm presence of the fusion construct insert. A photograph of an agarose gel showing the presence in seven plasmid clones of a 1.5kb insert released by NdeI/NotI digestion is shown in FIG. 3.

Following identification of insert-positive plasmid clones, the clones are sequenced, using standard techniques, across the NdeI juncture between the expression vector and the DNA sequence of the H2Ld ECD to confirm the absence of the signal peptide sequence and the presence of the correct amino terminal coding sequence. An automated sequencer sequence trace

from sequencing of a clone with the correct amino terminal coding sequence is shown in FIG. 4.

## EXAMPLE 2

### Expression of the H2Ld-DsRed fusion protein in bacteria

Using standard techniques, five clones of the H2Ld-DsRed fusion construct expression vector are used to transform competent *E. coli* bacteria containing an IPTG inducible T7 RNA polymerase. After growth overnight on ampicillin-LB agar plates, colonies of transformed cells are picked and expanded by growth overnight in LB media. The next day, the overnight culture is further grown at 37°C in fresh LB media containing IPTG (final concentration 1 mM) for 2 hours.

Samples of bacteria from induced cultures are then withdrawn and lysed in an SDS-containing buffer, after which the constituent proteins are separated by SDS denaturing polyacrylamide gel electrophoresis (PAGE). After electrophoresis, gels are stained with Coomassie stain, after which the separated, stained proteins are illuminated by visible light and photographed with a video still camera for visual analysis.

As shown in FIG. 5, for each of five H2Ld-DsRed fusion construct expression vectors used to transform *E. coli*, induction with IPTG results in expression of a 60kD protein, which matches the expected molecular mass of the H2Ld-DsRed fusion protein. In contrast, the induced 60kD band is absent from negative controls that were not IPTG-induced (FIG. 5).



As shown in FIG. 6, the degree of IPTG-dependent induction is dependent on the strain of E. coli transformed with a H2Ld-DsRed expression vector: the "NovaBlue" and "Origami" strains show strong IPTG  
5 induction of a 60kD protein. Using standard techniques, Western blot analysis of the gel stained with an anti-DsRed antibody confirms that the 60kD band contains DsRed protein (data not shown).

### EXAMPLE 3

10 Expression of the H2Ld-DsRed fusion protein in insect cells

The H2Ld-DsRed expression vector is co-transfected with BaculoGold™ DNA (BD-Pharmingen, Cat. No. 554739, formerly Cat. No. 21100D) into Sf9 and Tni  
15 insect cells using standard techniques discussed in the BD BaculoGold™ Linearized Baculovirus DNA technical data sheet, incorporated herein by reference in its entirety, and available on the Pharmingen website (<http://www.bdbiosciences.com/pharmingen/>). The  
20 expression vector serves as a transfer vector that complements a deletion in the BaculoGold™ virus DNA such that infectious virus is produced that contains within its genome the DNA encoding the H2Ld-DsRed fusion protein. Three days after transfection  
25 performed using standard techniques, medium from the transfected cell cultures is collected, cells are removed, and the supernatant is used in the first of three rounds of amplification to obtain a high titer stock solution of infectious baculovirus.

30 Using standard techniques, the high titer stock is then used to infect Sf9 and Tni cells for the

production of recombinant H2Ld-DsRed protein. Infected cells are solubilized with SDS-buffer and analyzed by SDS-PAGE, as described above for transformed and IPTG-induced bacteria. As shown in FIG. 7, a 60kD protein band is expressed by infected Sf9 and Tni cells, but not by negative control, non-infected cells. Using standard techniques, Western blot analysis of the gel stained with an anti-DsRed antibody confirms that the 60kD band contains DsRed protein (data not shown).

10

#### EXAMPLE 4

Fluorescence detection of DsRed in H2Ld-DsRed infected insect cells

Using standard techniques of fluorescence microscopy, insect cells infected with the H2Ld-DsRed fusion construct recombinant baculovirus produced as described in Example 3 are analyzed for expression of the H2Ld-DsRed fusion protein. Infected cells that produce H2Ld-DsRed fusion protein containing a functional DsRed chromophore are expected to emit red light by fluorescent emission.

FIG. 8 shows negative control, non-infected Sf9 cells after three days of growth in culture by conventional light microscopy. As shown in FIG. 9, negative control Sf9 cells produce no red fluorescent emission when stimulated and analyzed by fluorescent microscopy three days post-infection.

In contrast, FIG. 10 shows Sf9 cells infected with H2Ld-DsRed fusion construct recombinant baculovirus by conventional light microscopy after three days of growth in culture post-infection. As shown in FIG. 11, infected Sf9 cells emit red

fluorescent light when stimulated and analyzed by  
fluorescent microscopy three days post-infection.  
Fluorescent emission of red light indicates that  
functional (and thus almost certainly tetramerized)  
5 DsRed chromophore is produced by the Sf9 cells infected  
with the H2Ld-DsRed fusion.

Similar results are seen with Tni insect  
cells. FIG. 12 shows Tni cells infected with H2Ld-  
DsRed fusion construct recombinant baculovirus by  
10 conventional light microscopy after three days of  
growth in culture post-infection. As shown in FIG. 13,  
infected Tni cells emit red fluorescent light when  
stimulated and analyzed by fluorescent microscopy three  
days post-infection. Fluorescent emission of red light  
15 indicates that functional DsRed chromophore is produced  
by the infected Tni cells.

All patents and publications cited in this  
specification are herein incorporated by reference as  
if each had specifically and individually been  
20 incorporated by reference herein. Although the  
foregoing invention has been described in some detail  
by way of illustration and example, it will be readily  
apparent to those of ordinary skill in the art in light  
of the teachings herein that certain changes and  
25 modifications may be made thereto without departing  
from the spirit or scope of the appended claims, which,  
along with their full range of equivalents, alone  
define the scope of invention.